(P001) Mice carrying O-GlcNAc transferase intellectual disability mutations show microcephaly, hyperactivity and memory defects

Florence Authier[1], Nina Ondruskova[2], Andrew T. Ferenbach[1], Alison McNeilly[2], Daan M.F. van Aalten[1,4,*]

[1]Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark, [2]Department of Paediatrics and Inherited Metabolic Disorders, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic, [3]Division of Systems Medicine, School of Medicine, University of Dundee, Dundee, UK, [4]Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee, UK

The O-GlcNAc post-translational modification of intracellular proteins is essential for embryogenesis, development and brain function. However, there is still a huge gap in our knowledge of how O-GlcNAcylation regulates protein function and associated pathways. An exciting new possible inroad into this is our recent discovery that patients with mutations in O-GlcNAc transferase (OGT) suffer from intellectual disability and (neuro)developmental delay (e.g. PNAS 2019), in a syndrome that we have defined as OGT-Congenital Disorder of Glycosylation (OGT-CDG, Eur.J.Hum.Gen. 2020). Although several OGT-CDG mutations have recently been reported, further linking O-GlcNAc to neuronal function, it is not understood how these mutations are mechanistically linked to the neuro-developmental deficits seen in the patients. We will present the first example of genome editing in mice to generate three independent lines that carry OGT-CDG mutations. Excitingly, these mouse are viable, unlike previously reported Ogt knock-out mice, allowing the first phenotypic characterization of a vertebrate model of OGT-CDG. Notably, these mice show changes in size and weight suggesting developmental delay as observed in patients. Additionally, we observe changes in O-GlcNAc homeostasis to compensate for loss of OGT catalytic activity in the brain. This is associated with microcephaly, behavioural and cognitive defects, including hyperactivity, anxiety, compulsive behaviour and altered spatial working memory – again recapitulating several of the symptoms in OGT-CDG patients. Taken together, this is the first example of phenotypes in mouse models of OGT-CDG reminiscent of patient symptoms resulting from pathogenic loss of OGT catalytic activity. These models will be an invaluable starting point to gain insight into OGT-CDG etiology, identify underlying mechanisms of the disease and provide a platform for evaluation of potential future treatment strategies.

(P002) Increase in complexity of plasma protein N-glycosylation is positively associated with coronary artery disease

Barbara Radovani[1], Frano Vučković[2], Aldo P. Maggioni[3], Ele Ferrannini[4], Roberto Latini[5], Janko Szavits-Nossan[6,7], Aleksandar Blivajs[8], Igor Šesto[6,7], Krešimir Štambuk[6,7,9], Lovorka Đerek[10], Diana Rudan[8], Gordan Lauc[2,11], Ivan Gudelj[1,2]

[1]Department of Biotechnology, University of Rijeka, [2]Genos Glycoscience Research Laboratory, [3]Heart Care Foundation ANMCO Research Center, [4]CNR Institute of Clinical Physiology, [5]Mario Negri Institute for Pharmacological Research, [6]Magdalena Clinic for Cardiovascular Disease, [7]Faculty of Dental Medicine and Health, J.J. Strossmayer University in Osijek, [8]Department of Cardiology, University Hospital Dubrava, [9]Faculty of Medicine, J.J. Strossmayer University of Osijek, [10]Clinical Department for Laboratory Diagnostics, University Hospital Dubrava, [11]Faculty of Pharmacy and Biochemistry, University of Zagreb,

Coronary artery disease (CAD) is the most common cardiovascular disease (CVD), resulting from chronic inflammation of the coronary arteries due to the formation of atherosclerotic plaques, and its presence is a significant marker of adverse cardiovascular (CV) events. A growing body of research suggests that alterations in plasma protein glycosylation are involved in the development of CVD through various mechanisms and have significant biomarker potential because of their sensitivity to changes that occur in the organism during inflammation-related conditions such as CVD. Our aim was to determine whether the N-glycome of total plasma proteins is associated with CAD, because N-glycans are known to alter the effector functions of proteins, which may enhance their inflammatory response in CAD. Therefore, in this study, we analysed the N-glycome of plasma proteins isolated from subjects with coronary atherosclerosis (CAD+) and from subjects with clean coronaries (CAD-), grouped based on computed coronary tomography angiography (CCTA). Proteins were denatured and enzymatically deglycosylated, and the released and fluorescently labelled N-glycans were analysed by ultra-high performance liquid chromatography based on hydrophilic interactions with fluorescence detection (HILIC-UHPLC-FLR). Results showed significant differences in plasma N-glycan composition in CAD. CAD+ participants showed a decrease in complex biantennary galactosylated N-glycans with core fucose and an increase in highly branched (tri- and tetra-antennary) sialylated N-glycan structures with terminal fucose. These features are associated with the inflammatory epitope sialyl Lewis X (sLeX), which is found on plasma proteins and is known to propagate their inflammatory properties. The obtained chromatograms shed light on the plasma protein N-glycome composition in CAD and on the biomarker potential of N-glycans in CVD. Overall, protein N-glycosylation emerges as a significant factor in CAD, with glycan-based biomarkers showing promise for predicting cardiovascular health.

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(P003) AAV9-based PMM2 gene replacement augments PMM2 expression and improves glycosylation in fibroblasts of patients with inherited phosphomannomutase 2 deficiency (PMM2-CDG)

Mianling Zhong[1], Bijina Balakrishnan[1], Aaron Guo[1], Kent Lai[1]

[1]*Division of Medical Genetics, Department of Pediatrics, University of Utah Spencer Fox Eccles School of Medicine,*

In humans, inherited deficiency of Phosphomannomutase 2 (PMM2) (aka PMM2-CDG, MIM# 212065) is a debilitating congenital disorder of glycosylation (CDG). PMM2-CDG has a prevalence as high as 1:20,000 in some studied populations, making it the most common CDG. Affected patients present with variable features ranging from isolated neurologic involvement to severe multi-organ dysfunction. However, liver abnormalities characterized by hepatomegaly and elevated aminotransferases, along with coagulation irregularities occur in almost all patients. In the absence of curative treatments and with a significant mortality rate before two years of age, not to mention the severe morbidity throughout life, it is imperative to explore novel, safe and effective therapeutic strategies. In this study, we hypothesize that AAV9-based *PMM2* gene replacement can restore significant PMM2 expression in patient cells and improve the long-term outcome of this disease.

To begin, we conducted *in vitro* studies by infecting patient and control fibroblasts with AAV9-based vector expressing human *PMM2* gene. The former patient fibroblast strain is compound heterozygous for R141H and N216I mutations, while the latter is compound heterozygous for R141H and E139K mutations. Although both patient strains expressed residual PMM2 activity (9.09% and 22.73%, respectively of control) and immuno-reactive PMM2 protein (36.36% and 14.85%, respectively of control), infection of the vector at MOI of 10,000 augmented PMM2 protein levels by 70.84% and 252.21%, respectively. This was accompanied by an increase in activity by 200.00% and 80.00%, respectively. However, based upon the data we obtained from control (infected vs uninfected), which showed an absolute 4.42% increase in PMM2 protein led to an absolute 40.91% increase in activity, we expect such increases in PMM2 proteins in the infected patient fibroblasts should result in much enhanced PMM2 activities. Therefore, our results suggested a potential dominant negative effect of the mutant enzyme subunits.

Moreover, augmentation of PMM2 activity in the patient fibroblasts improved staining by Wheat Germ Agglutinin (WGA) up to 82.62% for selected glycoproteins, which indicated that *PMM2* gene replacement could correct glycosylation defects in the patient cells. Although we found a dose-dependent increase of PMM2 expression when we raised the MOI to 30,000, supplementation of the patient cells with 1mM D (+) mannose did not appear to improve the infection efficiency and WGA staining, which could have important implications in the pathophysiology and treatment strategy in the future. In conclusion, AAV9-based *PMM2* gene replacement is a promising therapeutic strategy to tackle the unmet medical needs for the patients with PMM2-CDG.

(P004) Laminin-211 mis-glycosylation activates MAP kinase signaling, impairs Pi3k/Akt pathway, and perturbs the membrane permeability in cardiomyocytes: Lessons from a mouse model of PGM1-CDG

Bijina Balakrishnan[1], Olivia Bellagamba[1], Sihem Boudina[2], Tamas Kozicz[3,4], Eva Morava-Kozicz[3,4], Kent Lai[1,2]

[1]*Division of Medical Genetics, Department of Pediatrics, University of Utah, Salt Lake City, USA,* [2]*Department of Nutrition and Integrative Physiology, College of Health, University of Utah, Salt Lake City, USA,* [3]*Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA,* [4]*Department of Clinical Genomics, Center of Individualized Medicine, Mayo Clinic, Rochester, USA*

Over 20% of patients with congenital disorders of glycosylation (CDG) present with heart failure. Yet, the cardiac diseases of CDG have not been fully explored despite their impact on morbidity and mortality. Here we examine the role of mis-glycosylation of **Laminin-211,** an extracellular matrix protein, in the development of dilated cardiomyopathy (DCM) in a mouse model of a CDG called inherited **Phosphoglucomutase 1 deficiency** (PGM1-CDG). While DCM is the key cause of mortality and morbidity for patients with PGM1-CDG, its pathobiology remains obscure and there is currently no effective therapy to relieve the disease burden. We have constructed a cardiomyocyte-specific *Pgm1*-cKO mouse model that mimic the patient phenotype at the functional, histological, and molecular levels. Initial glycoproteomic analysis unveiled broad glycosylation changes in the different subunits of Laminin-211. Immunoblots and immunohistochemistry showed decreased abundance of Laminin-211 protein in the *Pgm1*-cKO mouse heart over time. Importantly, such decrease in protein abundance was not accompanied by diminished gene expression, thus supporting that the reduced Laminin-211 is caused by post transcriptional mechanisms like mis-glycosylation. In addition to stabilize the basement membrane and muscle fibers during contraction., Laminin-211 also regulates diverse cellular functions through binding to the Dystrophin-Glycoprotein Complex (DGC) and the Integrin complex. Interestingly, mis-glycosylation of α-dystroglycan, part of the DGC, causes muscular dystrophies in humans. We hypothesize

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that mis-glycosylation of Laminin-211 causes its destabilization and adversely affects its ability to interact with Integrin and/or α-dystroglycan, thus contributing to the DCM in the mouse model of PGM1-CDG.

To support our hypothesis, we found a 70% decrease in the phosphorylation of Akt (Ser473) (a pro-survival factor) and its downstream effector Gsk3β in the mutant mouse hearts. Moreover, the mutant hearts exhibited a 2-fold activation of the extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinases (JNKs) branch of the mitogen-activated protein kinase (MAPK) pro-apoptotic signaling cascade. Yet, p38 kinase arm of MAPK signaling was unaltered unlike the case of Utrophin-dystrophin-KO mouse model. As Laminin-DGC complex is thought to maintain muscle membrane stability, we checked for abnormal changes in membrane permeability by Evans Blue dye uptake assay. We found a decrease in membrane permeability characterized by an increase in Evans Blue dye-positive cardiomyocytes in the mutant mouse hearts. Together, our results provide the first direct evidence that mis glycosylation of Laminin-211 is associated with the DCM development in PGM1-CDG. The unique findings could provide new insights into the cardiac pathogenesis of other CDG's, thus providing new therapeutic targets.

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(P005) Paradoxical expression of R-10G-reactive antigen in human testicular embryonal carcinoma

Motohiro Kobayashi[1], Akifumi Muramoto[1], So Inamura[1], Hitomi Hoshino[1], Naoki Terada[1]

[1]*Faculty of Medical Sciences, University of Fukui,*

Thus far, several monoclonal antibodies directed against cell surface carbohydrate antigens have been generated. Among them, R-10G, which binds to *N*-acetylglucosamine (GlcNAc)-6-*O*-sulfated di-*N*-acetyllactosamine (di-LacNAc) tetrasaccharide, a component of low-sulfated keratan sulfate, reportedly reacts selectively with human embryonic stem (ES) and induced pluripotent stem (iPS) cells, but not with embryonal carcinoma (EC) cells. However, EC cells derived from patients' EC tumors may exhibit varying levels of R-10G-reactive antigen expression. Thus, we asked whether human EC tissues or germ cell tumor (GCT) tissues other than EC express R-10G-reactive antigen. To do so, we quantitatively analyzed R-10G-reactive antigen expression in 83 testicular GCT surgical specimens containing a total of 125 various GCT components. Accordingly, in all EC components examined, the EC cell plasma membrane was immunolabeled with R-10G, while most seminoma components were R-10G-negative. In non-seminomatous GCT (NSGCT) other than EC (non-EC NSGCT), R-10G-reactive antigen expression was variable, but signal distribution was focal and the average intensity was weaker than that seen in EC. The percentages of R-10G-positive cells in these three groups varied with high statistical significance ($P < 0.001$ for all combinations). These findings indicate that the R-10G-reactive antigen is preferentially expressed in human testicular EC tissues and, thus, could be used as a diagnostic marker for this malignancy.

(P006) Intact glycoproteomics reveals protein glycoform signatures of Alzheimer's disease

Qi Zhang[1], Cheng Ma[2], Sheng Pan[2], Lih-Shen Chin[1], Lian Li[1]

[1]*Emory University School of Medicine,* [2]*University of Texas Health Science Center at Houston,*

Alzheimer's disease (AD) is a devastating neurodegenerative dementia with no effective treatment, highlighting the need for the use of unbiased molecular profiling approaches to discover novel targets for therapeutic intervention. Glycosylation is the most prevalent form of protein modification which produces vastly diverse arrays of glycosylated proteoforms or glycoforms to regulate many biological processes, including brain function. However, our current knowledge of human brain glycoforms and their alterations in AD is still limited. In this study, we established an integrated approach that combines intact glycopeptide-based glycoproteomics and systems biology for large-scale, in-depth analysis of glycoforms and site-specific N-glycan modifications in human brain and their changes in AD. We used this approach to analyze human brain tissue samples from neuropathologically confirmed AD cases and their age-matched controls. Our analyses identified over 10,000 human brain N-glycoforms from nearly 1200 glycoproteins and uncovered previously unknown disease signatures of altered glycoforms and glycan modifications in AD brain. Intact glycoproteomics data-driven network analysis revealed protein glycoform co-regulation networks in human brain and their disease-associated changes in AD. Our findings provide new molecular and systems-level insights into the roles of glycan modifications in AD pathogenesis and establish a new framework of glycosylation-based targets and networks for understanding and treating AD.

(P007) High Throughput Drug Discovery for Multiple Hereditary Exostoses

Kimberly Cisneros[1], Madison Intemann[1,2], Haruki Takeuchi[1,2], Antoinette C. Russell[3], Christopher Rice[4], Ryan J. Weiss[1,2]

[1]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA,* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA,* [3]*Center for Tropical & Emerging Global Diseases, University of Georgia, Athens, GA, USA,* [4]*Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine, West Lafayette, IN, USA*

Multiple Hereditary Exostoses (MHE) is a congenital skeletal disorder that is defined by the formation of cartilage-capped bony tumors, also known as exostoses, at the growth plate of long bones and other skeletal elements. This disorder affects one in every 50,000 children worldwide and currently there are no FDA-approved treatments. More than 90% of cases are caused by loss-of-function heterozygous mutations in exostosin-1 (*EXT1*) and exososin-2 (*EXT2*). These two genes encode for glycosyltransferase enzymes that are responsible for the biosynthesis of the cell surface polysaccharide, heparan sulfate (HS). Heterozygous mutations in either gene leads to a decrease in overall HS levels leading to the disruption of multiple signaling pathways responsible for regulating chondrocyte organization and function at the growth plate. Since a deficiency in HS is a key component of MHE, we hypothesize that overexpression of the normal *EXT* allele will compensate for the mutant allele and could restore functionally normal levels of HS in cells. Thus, the goal of this project is to utilize drug screening assays to identify small molecule agents that increase the expression of EXT1 and/or EXT2 as a form of treatment for MHE. We engineered EXT1 and EXT2 reporter cell lines and performed drug repurposing screens to search for FDA-approved drugs that increase the expression of EXT*1*/EXT2. From these screens, we have identified compounds that target diverse cellular pathways and enhance *EXT1* and/or *EXT2* expressions. We have validated the top hits by assessing alterations in HS biosynthesis, *EXT* expression, and chondrogenesis. In summary, this project utilizes drug screening assays to identify promising novel compounds that will hopefully bring us closer to finding a cure for MHE.

(P008) Drug Discovery for Sanfilippo Syndrome

Amrita Basu[1], Madison Intermann[2], Barry C. Maloney[2], Kimberly Cisneros[2], Antoinette C. Russell[3], Christopher A. Rice[4], Ryan J. Weiss[1,2]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602,* [2]*Department of Biochemistry and Molecular Biology, University of Georiga, Athens, GA 30602,* [3]*Center for Tropical & Emerging Global Diseases, University of Georgia, Athens, GA, USA,* [4]*Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine, West Lafayette, IN, USA*

Mucopolysaccharidosis Type IIIA (MPS IIIA), known as Sanfilippo Syndrome Type A, is an inherited lysosomal storage disorder wherein patients are unable to catabolize cellular heparan sulfate, due to inherited loss-of-function mutations in the gene encoding for the lysosomal enzyme, *N*-sulfoglucosamine sulfohydrolase (*SGSH*). Mutations in this enzyme lead to intra-lysosomal storage and accumulation of HS, which results in severe neuropathology, including regression of intellectual and motor abilities, behavioral problems, hearing loss, and dementia. Children born with this disorder exhibit developmental abnormalities, organ failure, and neurodegeneration, which often result in death within the first two decades of life. Unfortunately, there are currently no FDA approved treatments for this disease. The main objective of this project is to identify therapeutic agents to lower the accumulation of HS in MPS IIIA as novel substrate reduction therapies. We hypothesize that small molecule agents that lower expression of the key HS biosynthetic enzyme, EXT1, could potentially reduce cellular HS levels and lysosomal accumulation in cells, thus restoring cell homeostasis. To test this, we established a high-throughput drug screening assay using CRISPR-engineered EXT1 reporter cells and a library of FDA-approved drugs (2,300 compounds) to search for agents that could decrease EXT1 expression. Primary selection of screening hits was based on a cutoff of $\geq$ 75% inhibition of EXT1 levels with minimal cytotoxicity. Many of the top hits from the screen were previously shown to cross the blood brain barrier and are FDA approved for other therapeutic uses. Intriguingly, a subset of the top hits resulted in a significant decrease in intracellular HS levels and lowered lysosomal storage, as measured by the lysosomal membrane protein, LAMP1, in patient-derived fibroblasts. Overall, these studies have identified novel small molecule agents to reduce lysosomal storage of HS and provide exciting targets to pursue for downstream drug development for MPS IIIA and related disorders.

(P009) Utilizing Causal X-Linked Intellectual Disability Variants to Gain Insight into the O-GlcNAc Transferase Enzyme

Johnathan Mayfield[1], Laura Holden[1], Trevor Adams[1], Hannah Stephens[1], Lance Wells[1]

[1]*Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, 30605,*

X-linked intellectual disability (XLID) occurs in 1 in 500 males in the US with many cases being of unknown genetic etiology. Our laboratory was the first to biochemically characterize variants of O-GlcNAc Transferase (OGT), the sole enzyme responsible

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for the O-GlcNAc modification onto thousands of nucleocytoplasmic proteins, that are causal for an intellectual disability syndrome termed OGT-Congenital Disorder of Glycosylation (OGT-CDG). Initial OGT-CDG causal variants were identified in the Tetratricopeptide repeat (TPR) domain of OGT. While no significant differences in biochemical characterization were observed for these variants, RNA-seq demonstrated alterations in genes regulating neurogenesis in CRISP/Cas9-edited male human embryonic stem cells. Further, we have established that TPR domain variants have altered protein–protein interactions. Currently, we are characterizing novel catalytic domain variants of OGT, T570A, Y835C, and A952V, discovered in new OGT-CDG families. Our studies have shown that all three variants are active O-GlcNAc transferases, but they all fail to recapitulate the activity of wildtype OGT. This finding is in alignment with the molecular modeling of the catalytic domain variants, which suggested the variants are likely Km variants for the donor sugar nucleotide, UDP-GlcNAc. Furthermore, one variant, Y835C, shows decreased expression in multiple systems suggesting the variant results in decreased stability of the enzyme. Thus, these variants likely result in hypoglycosylation of key substrates. We are currently working to fully characterize the variants in regards to enzymatic activity towards protein and peptide substrates as well as the stability defects of Y835C. Surprisingly, both TPR and catalytic domain variants present similarly in OGT-CDG patients. Thus, we are currently pursuing the hypothesis that all variants, despite different altered biochemical characteristics, have a common downstream impact on glycosylation of key transcriptional regulators. Overall, this project aims to provide insight into how these OGT-CDG variants in different domains of OGT impact function of the enzyme and lead to the same neurodevelopmental phenotype observed in patients.

(P010) Exploring the Regulatory Mechanisms of EXT1 and EXT2 for Identifying Drug Targets in Multiple Hereditary Exostoses (MHE)

Xiaolin Dong[1], Kavya Suryadevara[2], Lance Wells[1,2], Ryan Weiss[1,2]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, 30605, USA,* [2]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30605, USA,*

Multiple Hereditary Exostoses (MHE) is an autosomal dominant skeletal disorder characterized by cartilage-capped bony outgrowths, also known as exostoses, that grow near the growth plate of long bones and other skeletal elements. MHE affects approximately 1 in 50,000 people worldwide, and there are currently no approved therapies. MHE patients present with several health issues, including skeletal deformities, chronic pain, short stature, and 1–5% of patients develop life-threatening chondrosarcoma. Approximately 90% of MHE patients have heterozygous loss-of-function mutations in exostosin-1 (*EXT1*) or exostosin-2 (*EXT2*), which are genes that encode for key glycosyltransferases involved in heparan sulfate (HS) chain elongation. A heterozygous mutation in *EXT1* or *EXT2* results in a ∼50% reduction of HS levels in cells. This disruption of HS biosynthesis affects multiple cell signaling pathways, such as BMP and MAP Kinase, which induces ectopic chondrogenesis and exostoses development. There is an urgent need to uncover the regulatory mechanisms of HS assembly and identify potential therapeutic targets for MHE. Our preliminary data suggest that upregulating the expression of EXT1 and/or EXT2 may restore HS levels in MHE cell models. The goal of this project is to uncover upstream regulatory factors that can enhance *EXT1* and/or *EXT2* expression as novel therapeutic targets for MHE. To achieve this, we have generated EXT1 and EXT2 reporter cell lines by integrating a green fluorescent protein (GFP) at the C-terminus of endogenous *EXT1* and *EXT2* in human chondrocytes using CRISPR/Cas9 gene editing. These reporter lines enable real-time monitoring of EXT expression. Subsequently, we developed a genome-wide CRISPR screening approach to search for regulatory factors that could be targeted to enhance EXT1 and/or EXT2 expression. Gene targets identified through the CRISPR screen, which enhance EXT expression and restore HS levels, hold significant promise as prospective drug targets of MHE. Moreover, genes identified through our CRISPR screening approach that downregulate EXTs offer an avenue for uncovering novel genes not previously linked to MHE pathogenesis.

(P011) A missense O-GlcNAcase mutation leads to reduced protein levels and intellectual disability

Florence Authier[1], Sergio Galan Bartual[1], Cyril Mignot[2], Boris Keren[2], Benedetta Attianese[1], Bartosz Kowalski[3], Conor Mitchell[1,2], Andrew Ferenbach[1], Diane Doummar[2], Perrine Charles[2], Daan van Aalten[1,2]

[1]*Aarhus University,* [2]*AP-HP Sorbonne Université,* [3]*University of Dundee,*

Intellectual Disability (ID) is characterised by substantial limitations in cognitive function and adaptive behaviour, affecting 1–3% of the population. Protein O-GlcNAcylation is a posttranslational modification of nucleocytoplasmic proteins regulated by two opposing enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). Recently, missense mutations in *OGT* have been shown to segregate with ID, associated with compensatory reduction of OGA protein expression. Human OGA (hOGA) is encoded by the meningioma-expressed antigen 5 (*MGEA5*) gene. There are two main splice variants of OGA: a long and a short isoform, named OGA-L and OGA-S respectively. The OGA-L isoform is composed of an N-terminal hydrolase domain, a central stalk linker domain and a C-terminal pseudo-histone acetyltransferase (pHAT) domain, whereas the short isoform completely lacks the pHAT domain. The pHAT domain shares similarities with histone acetyl transferases but lacks the key amino acids required for catalysis. While OGA catalytic domain and activity have been well characterized, the role of the pHAT domain

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remains unknown. Here, we report a patient with a *de novo* missense mutation in the pHAT domain of OGA, associated with ID, infantile spasms and autism. While the mutation does not affect protein stability or activity *in vitro,* mouse embryonic stem cells edited to harbour the mutation show reduced OGA levels associated with increased turnover. Taken together, these data establish a possible link between missense mutations in OGA and ID.

(P012) Platelet and Myeloid Cell Phenotypes in a Rat Model of Fabry Disease and the Role of Glycosphingolipids in Sensitizing Platelets to Agonist-induced Activation

Eve Prodoehl[1], Adam J. Kanack[1], Mayumi Ishihara-Aoki[1], Kazuhiro Aoki[1], Nancy M. Dahms[1]

[1]*Medical College of Wisconsin,*

Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of the lysosomal enzyme α-Galactosidase-A (α-GalA). Fabry disease results from the widespread accumulation of the glycosphingolipids (GSLs) globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3), impacts multiple organ systems and leads to impaired quality of life. Thrombotic events are common, with strokes and heart attacks contributing to a shortened lifespan for male and female Fabry patients. Previously, we showed GSL accumulations in the bone marrow, circulation, and platelets from male α-GalA-deficient rats contribute to increased platelet activation in response to agonists. However, the extent that these GSLs accumulate in the female Fabry population and the mechanisms by which Gb3 and lyso-Gb3 increase thrombotic risk are incompletely defined. To further define the mechanisms linking GSL accumulation to thrombotic disease, we used mass spectrometry (MS), platelet-based assays, and histological characterization of our α-GalA-deficient animal model. To assess for changes in platelet sensitivity and aggregation in response to platelet agonists, we used flow cytometry to quantify fibrinogen binding to the integrin GPIIb/IIIa or light-transmission aggregometry after agonist treatment of platelets isolated from α-GalA-deficient female rats. We found that α-GalA-deficient females were not sensitized to increased platelet activation or platelet aggregation in response to ADP. However, analysis of serum and platelets from male and female α-GalA-deficient rats by MS showed drastic differences in GSL profiles compared to wild-type (WT) rats, and the bone marrow of 52-week-old females stained with *Griffonia simplicifolia isolectin* B4 (IB4) revealed striking accumulations of GSLs. Complete blood counts also revealed that 15–75 week-old, homozygous Fabry female rats had significant increases in lymphocyte and monocyte counts compared to WT females, indicating chronically high levels of white blood cells in their circulation. Lastly, we assessed the ability of α-GalA substrates to sensitize platelets to activation by incubating platelets isolated from 50–75-week-old WT male and female animals with various concentrations of either Fabry-associated or control (i.e., lack a terminal galactose residue) GSLs. Notably, WT platelets incubated with GSLs that accumulate in Fabry patients (10μM Gb3 and 2μM lyso-Gb3, or 10μM Gb3 alone), as opposed to control GSLs (10μM glucocerebroside and 2μM lyso-glucocerebroside), stimulated increased baseline platelet activation, fibrinogen binding, and platelet aggregation, even in the absence of exogenous platelet agonists. Together, these data suggest that chronically high concentrations of the Fabry-associated GSL, Gb3, can directly contribute to increased baseline platelet activation and aggregation. (NIH K12HL141954 to AK and NMD, R01DK042667 to NMD).

(P013) Role of glycosylation in thrombocytopenia and acute myeloid malignancies associated with RUNX1 Familial Platelet Disorder

Débora Andrade Silva[1], Jennifer Kohler[1]

[1]*University of Texas Southwestern Medical Center,*

Congenital disorders of glycosylation (CDG) are frequently associated with problems in the coagulation process, highlighting the importance of this modification for hemostasis. This observation opens the possibility of other congenital disorders presenting as hematological deficiencies that could be related to glycosylation defects but that are still not understood. In this project, we focus on glycosylation defects in RUNX1 familial platelet disorder (RUNX1-FPD), a hereditary condition that affects platelet production and that is a predisposition to leukemia development. RUNX1 is a transcription factor related to hematopoiesis, which has been implicated in the orchestration of sialyltransferase expression. Therefore, we hypothesize that a hyposialylation state is part of the mechanism involved in RUNX1-FPD. The general aim of this project is to explore if RUNX1-FPD is related to glycosylation defects. To address this, we generated a RUNX1 knockout K562 cell line, and the preliminary data indicated that these cells have a decrease of α2,3 sialylation but the same level of α2,6 sialylation. These data agree with the literature reports indicating RUNX1 as an important orchestrator of sialyltransferases involved in adding sialic acid at position α2,3. The following steps of the project include analysis of sialylation levels using DMB derivatization, selective periodate oxidation and aniline-catalyzed oxime ligation method (PAL) to confirm the sialylation levels. Furthermore, we will use a 2,3 sialidase to explore the effects of decreasing this specific type of sialylation on myeloid cells. In the end, we will investigate whether changes in myeloid cell glycosylation affect recognition by Siglecs. The results of this project will expand the knowledge available regarding the function of glycosylation in RUNX1-FPD, an unexplored perspective of this disease, and explore the role of sialylation in hemostasis and leukemia development.Support: Mary Kay Charitable Foundation, NIH R35GM145599.

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(P014) Mechanistic Insights into LARGE1 Elongation of Matriglycan on Dystroglycan: Implication for Therapies

Kevin Campbell[1]

[1]*Howard Hughes Medical Institute, Department of Molecular Physiology and Biophysics, Carver College of Medicine, The University of Iowa,*

Matriglycan is a linear repeating disaccharide of alternating xylose and glucuronic acid that serves as a scaffold in many tissues for extracellular matrix (ECM) proteins containing laminin-G domains, including laminin, agrin, and perlecan. Like-acetylglucosaminyltransferase-1 (LARGE1) uniquely synthesizes and extends matriglycan on α-dystroglycan (α-DG). Abnormalities in the post-translational processing of α-DG that result in the absence or reduction of matriglycan cause various forms of muscular dystrophy, known as dystroglycanopathies, with or without brain and eye involvement. Interestingly, patients with dystroglycanopathies that exhibit milder symptoms have less or shorter forms of matriglycan although the clinical significance of this is unclear. Generation of full-length mature matriglycan on α-DG ($\sim$150 kDa) requires phosphorylation of core M3 by POMK. We previously used a multidisciplinary approach to show that the absence of POMK activity does not preclude the addition of matriglycan. Instead, in the absence of phosphorylation of core M3 by POMK, LARGE1 synthesizes a noticeably short, non-extended form of matriglycan on α-DG ($\sim$100 kDa). We now show that the N-terminal domain on dystroglycan (α-DGN) enables LARGE1 to extend matriglycan on α-DG and prevent muscular dystrophy. Using muscle from a mouse model lacking α-DGN, we find that LARGE1 can synthesize a short, non-elongated form of matriglycan, which results in a $\sim$100 kDa α-DG. This short form of matriglycan binds to laminin and maintains specific force but does not prevent muscle pathophysiology, including damage induced by lengthening contractions and abnormalities in neuromuscular junctions. Our current working hypothesis is that phosphorylated core M3 and α-DGN together anchor the LARGE1 onto α-DG to form an enzyme-substrate complex for full-length matriglycan synthesis. Collectively, our work provides insights into how full-length matriglycan is synthesized so it can function as a scaffold for ECM proteins, thereby enabling proper skeletal muscle function and preventing muscular dystrophy.

(P015) S6 domain in the N-terminal of Dystroglycan is required for synthesis of full length matriglycan

Ishita Chandel[1], Kevin P. Campbell[1]

[1]*HHMI/University of Iowa,*

Matriglycan is a linear heteropolysaccharide of alternating glucuronic acid and xylose units synthesized on α-Dystroglycan (α-DG) and is essential for neuromuscular function and brain development. It binds extracellular matrix proteins that contain laminin globular domains such as laminin and serves as a receptor for Old-world arenaviruses such as Lassa fever virus. It is synthesized on α-DG by the Golgi enzyme, like acetylglucosaminyltransferase-1 (LARGE1). Recently, it has been reported that the N-terminal domain of α-DG (α-DGN) is required to generate a full length matriglycan on α-DG. A short, non-elongated form of matriglycan is unable to prevent muscular dystrophy. It is speculated that α-DGN enables binding of α-DG to the enzyme LARGE1 which is then able to extend the matriglycan chain. However, it is unclear how α-DGN facilitates this binding. We have previously reported a mutation in the S6 domain of α-DGN, T192M which was associated with limb girdle muscular dystrophy, showed reduced laminin binding and a reduction in the size of matriglycan. We found another possible mutation in the S6, D196V reported in ClinVar database associated with limb girdle muscular dystrophy type 2P. There is no reported functional significance for this variation. Here, we report that similar missense mutations in α-DGN in mice (D196N and D194N) lead to a shorter matriglycan and reduced laminin binding. Missense mutations in immunoglobulin like (IG1) domain of α-DGN in mice did not affect laminin binding or the size of matriglycan or dystroglycan. These data suggest that the S6 region of α-DGN is important for enabling matriglycan elongation and possibly for binding to LARGE1. The D196 residue is moderately conserved and the function of S6 is currently unknown. Hence this study may shed light into the function of S6 and provide insight into how α-DGN enables binding of α-DG to LARGE1.

(P016) Glycosylation alterations in response to treatment with antidiabetic agents

Tamara Štambuk[1], Nikol Mraz[1], Frano Vučković[1], Tea Pribić[1], Dario Rahelić[2], Gordan Lauc[1,3]

[1]*Genos Glycoscience Research Laboratory,* [2]*University Clinics for Diabetes, Endocrinology and Metabolism, School of Medicine, University of Zagreb, Zagreb, Croatia,* [3]*University of Zagreb Faculty of Pharmacy and Biochemistry, Zagreb, Croatia,*

Type 2 diabetes represents a significant global public health challenge, with its prevalence steadily increasing. Extensive research over past decades has led to the development of new medications aimed at improving disease management. However,

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timely initiation of appropriate treatment is equally crucial for reducing disease-associated long-term complications, morbidity, and mortality. Many patients with type 2 diabetes are prescribed medications such as metformin, glucagon-like peptide-1 receptor agonists (GLP-1), sodium-glucose co-transporter-2 (SGLT2) inhibitors, and insulin. Still, some ambiguity regarding the mechanisms of action and patient responses to these agents remains. Given that glycans play a role in the majority of physiological processes, the use of these medications might be linked to distinct glycosylation patterns. Thus, we aimed to identify the associations of the immunoglobulin G (IgG) glycosylation with the use of the aforementioned agents, in patients with type 2 diabetes, in a longitudinal manner. IgG was isolated from the plasma samples by affinity chromatography. IgG N-glycans were enzymatically released, fluorescently labelled and analyzed by hydrophilic interaction ultra performance liquid chromatography (HILIC-UPLC). Study population included patients with type 2 diabetes starting the therapy either with metformin (N=44), insulin (N=5), GLP-1 agonists (N=23) or SGLT2 inhibitors (N=53). All patients were sampled at baseline, and every 3 months, up to the period of one year. Linear mixed models were used to investigate associations between glycans and medication use, adjusted for age and sex, and corrected for multiple comparisons. Preliminary results have showed the extensive changes in the N-glycome composition related to the introduction od antidiabetic therapy. Precisely, the use of GLP-1 agonists and SGLT2 inhibitors induced an increase in IgG mono- and digalactosylation, which are the IgG glycome changes previously associated with cardioprotective effects. Moreover, an increase in IgG fucosylation was observed in all patient groups, while the use of GLP-1 agonists was associated with a decrease in total sialylation. No significant changes were observed with respect to the insulin use. In conclusion, the introduction of antidiabetic therapy induced marked changes in the IgG N-glycome composition, but the current study design could not reveal the causality of these associations. Nonetheless, the use of such agents should be taken into account as a potential confounder when designing a glycomic study.

(P017) In-depth analysis of on-slide tissue glycome and proteome of young and old mouse brains using data independent acquisition mass spectrometry analysis

Sayantani Chatterjee[1], Shira Freilich[1], Margaret Downs[1], Joseph Zaia[1,2], Manveen K. Sethi[1]

[1]Department of Biochemistry, Center for Biomedical Mass Spectrometry, Boston University Chobanian & Avedisian School of Medicine, Boston, Massachusetts, USA, [2]Boston University Bioinformatics Program, Boston University, Boston, Massachusetts, USA,

Background and Aims: Neurodegenerative diseases are often associated with poor prognosis. Detected at advanced stages with limited treatments, the mechanism of these diseases remain poorly understood. Investigation for biomarkers specific to diseased brains can allow for early detection on the onset of disease as well as for improved therapeutics. A previously overlooked source of brain biomarkers is the dynamic and ever-changing extracellular matrix (ECM). ECM is heavily decorated with glycoproteins and proteoglycans that play several functions including cell differentiation, synaptic plasticity, and structural support. In-depth glycomics and proteomics profiling of aging brains can be a valuable resource to identify significant risk factors in development of neurodegenerative diseases. This study aims to improve the currently established on-slide digestion method to retrieve disaccharides and peptides by utilizing advanced instrumentation and a data-independent acquisition liquid chromatography-tandem mass spectrometry (DIA-LC-MS/MS) approach.

Study Design and Research Methods: Frozen whole brains of 12 wild-type mice (commercial, BioIVT), divided into two groups based on gender (n=6 for male/female) and age (n=6 young/old), were sectioned into 3 serial sections with a thickness of 10 μm. A 4 mm diameter region from the center of each brain was digested with chondroitinase ABC, heparin lyses, and trypsin successively to extract chondroitin sulfate disaccharides, heparan sulfate disaccharides, and peptides respectively. Desalted disaccharides were analyzed using a HILIC-LC-QTOF-MS/MS system and manually analyzed using Agilent Qualitative Analysis vB.06. Desalted peptides were acquired using a C18-LC-Orbitrap Fusion Lumos Tribrid-MS/MS system in both data-dependent acquisition (DDA) and data-independent acquisition modes. Proteomics data were analyzed and label free quantitation was performed using the Peaks Xpro/PeaksQ software, respectively. Statistical analyses for both disaccharides and peptides were performed using the R-package.

Results and Conclusions: For DIA-proteomics analysis, a total of 1,775 proteins and 13,670 peptides were found in comparison to DDA that identified only 1,309 proteins and 11,832 peptides. A total of 555 unique proteins (~2 unique peptides), including 31 (13 unique for DIA) glycosylated ECM proteins, were identified. Annexins (A11, A3, A7), glypican-1, disintegrin, metalloproteinases, laminin beta-2, and tenascin were identified from the mouse brains using the DIA-LC-MS/MS approach and differences in profiles between young and old brains with gender-biases were established. Thus, the on-slide digestion method when coupled with the DIA-approach has confidently shown to yield higher protein identification and this systematic workflow has aimed to generate a comprehensive proteomics and glycomics map of age- and gender-matched mouse brains that can provide crucial insights into the mechanisms of aging brains.

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(P018) Variants in sialic acid biosynthetic gene *GNE* associated with severe pediatric congenital thrombocytopenia

Katelyn Rosenbalm[1], Agata Steenackers[1], George F. Steinhardt[1], Anna P. Schmidt[1], Barbara Zieger[2], Doris Boeckelmann[2], Karin M. Hoffmeister[1,3]

[1]*Versiti Blood Research Institute,* [2]*University of Freiburg,* [3]*Medical College of Wisconsin,*

Platelets, crucial for hemostasis, are derived from bone marrow megakaryocytes and have a limited life span of ten days in humans. Platelet production and clearance are tightly controlled processes to maintain a steady state platelet of 150–450 x 109/L. Low platelet count (thrombocytopenia) can be mild and asymptomatic or lead to life-threatening internal bleeding resulting in death. The primary therapy for thrombocytopenia remains platelet transfusion. Congenital thrombocytopenia is characterized by decreased production and accelerated removal of platelets from circulation. The molecular mechanisms controlling platelet production and removal have yet to be fully understood. Evidence supports that platelet life span is determined partly by sialic acid decorations, which protect from clearance via the hepatic Ashwell-Morell receptor.The *GNE* gene encodes a bifunctional enzyme that initiates and regulates the biosynthesis of sialic acids. *GNE* variants are autosomal recessive with adult-onset progressive *GNE* myopathy and autosomal dominant with sialuria. In recent years, biallelic *GNE* mutations, found mainly in the N-acetylmannosamine (ManNAc) kinase domain, have been associated with isolated thrombocytopenia in pediatric patients.Recently, we identified compound heterozygous *GNE* variants in a pediatric female (P1) with life-threatening intracranial bleeding and severe congenital thrombocytopenia (lowest platelet count 5 x 109/L) with severely reduced platelet function needing weekly platelet transfusions. Our data demonstrated decreased binding of α2,3 sialic acid and increased terminal galactose and α2,6 sialic acid moieties in platelets. She underwent hematopoietic stem cell transplantation (HSCT), normalizing platelet count and glycan composition. Additional biallelic *GNE* variants in two siblings (P2 and P3) with congenital thrombocytopenia (platelet count 20 x 109/L and 50 x 109/L, respectively) showed severely decreased thrombin-induced platelet CD62 and CD63 exposure as determined by flow cytometry, suggesting impaired α- and δ-granule secretion and reduced platelet function. Lectin binding analysis showed decreased α2,3 sialylation (MAA) and increased terminal galactose (RCA120) expression on platelets. The data is consistent with loss of sialic acid synthesis and indicative of rapid platelet clearance. P4 (platelet count 50 x 109/L), possessing a homozygous variant of the UDP-GlcNAc epimerase domain, displayed reduced α2,3 sialic acid and compensatory glycan capping on platelets with increased fucose binding. Thrombocytopenia induced by *GNE* mutations opens a new avenue for investigating the role of sialic acids in platelet longevity and functionality.

(P019) Loss of sialic acid side-chain O-acetylation exacerbates colitis

Shubham Parashar[1], Yang Ji[1,2], Yukiko Miyamoto[3], Saurabh Srivastava[1,2], Nissi Varki[1,2], Lars Eckmann[3], Ajit Varki[1,2]

[1]*Glycobiology Research and Training Center, University of California San Diego, La Jolla, CA 92093, USA,* [2]*Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA 92093, USA,* [3]*Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA,*

Sialic acids (Sias) are nine-carbon backbone monosaccharides adorning cell surface & secreted glycoconjugates, modulating processes ranging from host-pathogen interactions to development. Among myriad of Sia modifications, O-acetylation is particularly common, introducing O-acetyl esters to hydroxyl groups on Sia C7/C8/C9 side-chain, enhancing hydrophobicity & resistance to sialidases. Physiological roles & structural intricacies these modifications impart across diverse animal tissues remain uncertain, primarily due to inherent chemical instability & generation of resistance to sialidases. Here, we explored functional implication of Cas1 domain-containing 1 (CASD1), only currently known Sialate: O-acetyl transferase, in modulating host-microbial interactions & susceptibility to inflammatory challenges. Using CASD1 KO mice in conjunction with sialoglycan-recognizing probes (SGRPs), histochemical profiling, & HPLC following fluorescent DMB labelling, we mapped distribution & dynamics of Sia modifications. CASD1 KO leads to elimination of Sia side chain O-acetylation across almost all mouse tissues, suggesting its central role in these modifications. In WT mice, colon had highest O-Ac Sias levels, followed by testis, heart, brain, & spleen. predominant O-Ac Sias in colon were C7-O & C9-O-Ac derivatives of Neu5Ac, with minimal C8-O-Ac & no 9-O-Ac Neu5Gc. Histochemical staining with SGRP9 & SGRP7, targeting C9-O-Ac Sia & C7/C9-O-diacetylated Sias, validated these findings. CASD1 KO mice lacked both C9-O-Ac & C7/C9-O-diacetylated Sias in the colon, but total Sia levels, especially 2-3-linked Sias remained unaltered, emphasizing CASD1's key role in Sia C7/C9-O-Ac modifications. Both conventional and germ-free mice had similar O-Ac Sias levels, with subtle differences in 7-O- & 9-O-Ac Sias expressions. Metagenomics analysis of fecal samples revealed that while CASD1 KO didn't have significant changes in overall microbial composition, they had significant differences in abundance of selected metabolic pathways. Notably, P441-PWY pathway genes, linked to N-acetylneuraminate degradation, were more abundant in CASD1 KO mice, highlighting link between Sia modifications & microbial metabolism. We also explored role of Sia O-acetylation in intestinal inflammation. Acute colitis induction by DSS feeding in WT mice led to depletion in C7-, C8-, C9-O-Ac-Sias & C7/C8, C9-O-diacetylated Sias, yet overall Sia levels changed minimally. Using

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SGRP9, we histologically observed reduced C9-O-Ac Sias in inflamed areas & a complete loss in ulcerated regions. Similar results were obtained in intestinal biopsies from IBD patients Importantly, CASD1 KO mice exhibited increased colitis severity in comparison to WT, indicating that Sia O-acetylation has a protective role in intestinal inflammation. Together, our studies show CASD1 is central for O-Ac Sias biosynthesis, affecting host-microbial interactions & contributes to intestinal protection against inflammatory insults.

(P020) PROBING O-MANNOSYLATED SITES VIA BIOORTHOGONAL SELECTIVE EXOENZYMATIC LABELLING (SEEL)

Ashley Carter[1], Linda Zhao[1], David Steen[1,3], Jeremy Praissman[1], David Live[1,3], Lance Wells[1,3], Geert-Jan Boons[1,2,4]

[1]Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA, [2]Department of Chemistry, University of Georgia, Athens, GA, USA, [3]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA, [4]Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, and Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands

Defects in O-mannosylation of α-dystroglycan (α-DG) lead to dystroglycanopathies, which are congenital muscular dystrophies involving neurodevelopmental abnormalities. Interactions between α-DG and its extracellular matrix (ECM) ligands require α-DG to be extended by O-mannose structures based on the M3 core structure by the glycosyltransferase POMGNT2. The core M3 can be extended with matriglycan, which is a repeating disaccharide that binds laminin globular domain containing proteins in the ECM. The only available detection methods for the M3 sites are antibodies that recognize the disaccharide repeats of matriglycan. Therefore, it remains a possibility that unextended M3 structures exist, but there is currently no tool available to identify core M3 glycans without the presence of the repeating disaccharide. A route for enhanced detection of theses glycans was developed by employing bioorthogonal tagging with an azido-modified form of UDP-GlcNAc and subsequentially clicking with a bioorthoganol biotin tag to enrich for modified protein sites. Leveraging bioorthogonal selective exoenzymatic labelling (SEEL), O-mannose sites were enriched for on the surface of living cells. The O-mannose sites were extended by POMGNT1 and POMGNT2, glycosyltransferases that mediate the branching point of the O-mannosylation pathway. Interactions between α-DG and its ECM ligands require α-DG to be extended by O-mannose structures based on the rare M3 core structure, which is highly selectively formed by POMGNT2 facilitating the addition of UDP-GlcNAc in a β-1,4 linkage. The core M3 can be extended with matriglycan, which is a repeating disaccharide that binds LG-domain containing proteins in the ECM. Previously, no method existed to identify the M3 core without the presence of matriglycan. The methodology described herein enriches for O-mannosylated probes independently of matriglycan. O-mannosylated sites were probed using the azido-form of UDP-GlcNAc and a bioorthogonal biotin tag. Along with known O-mannnosylated proteins α-DG and KIAA1549, APMAP and LAMB1 were also enriched and identified as potentially novel O-mannosylated sites.

(P021) Non-POMT1/2 O-Mannosylated Proteins Avoid POMGNT1 Extension

David Steen, Jr.[1], Sally Riewe Boyd[1], Lorenzo Povolo[2], Adnan Halim[2], Lance Wells[1]

[1]University of Georgia, Complex Carbohydrate Research Center, [2]University of Copenhagen, Copenhagen Center for Glycomics,

Protein O-linked mannose beta-1,2-N-acetylglucosaminyltransferase 1 (POMGNT1) is a GlcNAc transferase that catalyzes the addition of GlcNAc to an O-linked mannose in the beta-1,2 position forming the core M1 structure. The M1 structure can be branched to form core M2 structures, and both can be further extended by enzymes in mammals. Despite recombinant POMGNT1 being promiscuous on multiple synthetic O-mannose peptides derived from alpha-dystroglycan (alpha-DG), multiple non-POMT1/2 O-mannosylated glycoproteins appear to only contain core M0 structures (i.e. the O-mannose monosaccharide) thus apparently avoiding extension by POMGNT1 in cells. These M0 modified glycoproteins include the cadherins and the plexins. In order to investigate this further, we expressed and purified all glycoproteins in HEK293F POMGNT1 KO cells: both known M0 O-mannosylated glycoproteins E-cadherin, N-cadherin, and c-MET as well as alpha-DG (to generate M0 structures at sites that would normally be M1). We confirmed that the M0 modified glycoproteins were indeed not extended and that the O-hexose observed via mass spectrometry was in fact an O-mannose based on mannosidase sensitivity. We also demonstrated that recombinant POMGNT1 extended O-mannose peptides derived from trypsin digests of all four of the glycoproteins. However, only alpha-DG served as an efficient acceptor substrate for POMGNT1 at the protein level. Given that O-mannosylation and protein folding occur in the ER while POMGNT1 is localized to the cis-Golgi, our results suggest a steric or electrostatic hindrance of POMGNT1 preventing it from acting on cadherins and plexins which is not present on alpha-DG.

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(P022) ALG13-CDG Brain Organoids Exhibit Glycosylation Defects and Altered Gene Expression, Providing Insights into Neuronal Pathology

Rameen Shah[1,2], Silvia Radenkovic[2], Rohit Budhraja[3], Alexia Tyler King[4], Charlotte Bleukx[2], Ibrahim Shammas[2], Akhilesh Pandey[2], Steven Sloan[4], Tamas Kozicz[2,3], Eva Morava[2,3]

[1]*Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN,* [2]*Department of Clinical Genomics, Mayo Clinic, Rochester, MN,* [3]*Department of Laboratory Medicine and Pathology , Mayo Clinic, Rochester, MN,* [4]*Department of Human Genetics, Emory University, Atlanta, GA*

N-linked congenital disorders of glycosylation (CDG) comprise a rapidly expanding group of rare metabolic disorders, impacting both patient quality of life and lifespan. Asparagine-linked glycosylation 13 (ALG13) is an X-linked CDG where most surviving patients are females as the disease leads to an early lethal phenotype in males. Missense variants in *ALG13* gene cause dysfunction in the glycosyltransferase 28 domain of the ALG13 protein. Interestingly, unlike numerous other CDG, investigations on patients' blood and fibroblast samples have not revealed significant defects in protein glycosylation in ALG13-CDG. ALG13-CDG prominently exhibits neurological symptoms—seizures, intellectual disability, hypotonia, and developmental delays. This has led us to hypothesize that ALG13-CDG might exert its effects in a brain-specific manner. To test this hypothesis, it was imperative to develop a brain model of ALG13-CDG. First, we generated induced pluripotent stem cells (iPSCs) from fibroblasts bearing variants within the glycosyltransferase 28 domain (c.320A>G, N107S) of the *ALG13* gene, alongside iPSCs derived from three age-matched control fibroblast lines. Subsequent steps involved differentiating cortical brain organoids (BOs) from these iPSCs. Individual BOs were subjected to comprehensive multiomics profiling using single-cell RNA-seq, proteomics, glycoproteomics, and metabolomics analyses at day 80 of differentiation. Our investigation revealed significant reduction in glycosylation of several proteins pivotal to axon growth, neuronal migration, and synaptic plasticity. Notably, a parallel reduction in glycosylation was observed for proteins implicated in seizure. The application of single-cell RNA-seq elucidated distinct clustering patterns among GABAergic neurons, glutamatergic neurons, astrocyte progenitor cells, interneurons, and radial glia with significant changes in gene expression of genes important to proper brain function. Furthermore, our metabolomics investigations highlighted an elevation in GlcNAc levels. Our multi-omics studies on ALG13-deficient BOs provide significant insights into specific brain-related disturbances inherent to ALG13-CDG and open promising avenues for potential therapeutic interventions.

(P023) Advanced glycation end products induced skeletal muscle atrophy involves RAGE-NFκB pathway and suppression of growth factors

Mariyam Khalid[1], Abdu Adem[1]

[1]*Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Khalifa University, Abu Dhabi P.O. Box 127788, United Arab Emirates,*

Glycation is relatively a slow naturally occurring reaction and a normal part of the aging process resulting in modifications primarily in long-lived macromolecules. This process becomes accelerated in certain conditions like diabetes due to persistent hyperglycemia. The accumulation of advanced glycation end products (AGEs) in diabetes has been linked to the pathogenesis of various complications associated with this condition, including skeletal muscle atrophy. Skeletal muscle atrophy is of multifactorial etiology and is characterized by various pathological conditions. Skeletal muscles are not only the fundamental site for glucose uptake and utilization but also the main protein reservoir in the body. The diminished skeletal muscle insulin sensitivity in type 2 diabetes mellitus activates proteolytic signaling pathways, decreases protein synthesis, and reduces myofiber size. However, a substantial knowledge gap persists regarding the intricate interplay between type 2 diabetes, AGEs, and muscle atrophy. This study aimed to bridge that gap.

We investigated AGEs accumulation, RAGE expression, nuclear factor kappa B (NF-κB) p65 unit, growth factors, forkhead box transcription factor (FoxO3A), and atrogenes in gastrocnemius muscles of streptozotocin (STZ-) induced chronic eight months diabetic male Wistar rats and their age-matched non-diabetic control rats. The diabetic rats showed marked increases in AGEs, RAGE expression, as well as upregulated NF-κB p65 unit as compared to non-diabetic rats. The results showed a parallel correlation between FoxO3A and transforming growth factor beta (TGFβ), and a marked increase in atrogin1 and myostatin expression in diabetic rats. While gastrocnemius muscle growth factors [insulin growth factor (IGF1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and epidermal growth factor receptor (EGFR)] were downregulated in diabetic rats. Histological analysis showed a significantly higher percentage area of collagen fibers as well as collagen density in the diabetic control group as compared to the non-diabetic control group.

Overall, the findings from this study reveal that prolonged diabetes and AGEs accumulation disrupt muscle metabolism, muscle mass, function, and its robust regenerative potential to recover from injury. These effects are attributed to the activation of the RAGE-NFκB signaling pathway, which triggers oxidative stress and the activation of the FoxO family of transcription factors. These transcription factors are central in orchestrating the catabolic response that contributes to muscle atrophy in

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the context of diabetes. These insights provide valuable pathways to understand the perturbations involved in diabetes-induced skeletal muscle atrophy which, in turn, may lead to the development of potential therapeutic approaches aimed at mitigating this condition.

(P024) Antiglycation, antioxidant and insulinotropic properties of Withania coagulans in vivo: Possible role in management of diabetic complications

Abdu Adem[1], Mariyam Khalid[1]

[1] *Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Khalifa University, Abu Dhabi P.O. Box 127788, United Arab Emirates,*

Diabetes mellitus is the oldest known disease with a history reaching back to ancient times. The noninsulin-dependent type 2 diabetes mellitus accounts for 90–95% of total cases with diabetes. The hyperglycemia-induced accumulation of advanced glycation end products (AGEs) is considered to be both the cause and effect of diabetes mellitus. AGEs play a significant role in the complications associated with diabetes by causing irreversible damage to the structural and functional integrity of proteins through intermolecular and intramolecular crosslinks. AGEs-mediated damage also occurs through interaction with the cell surface receptor RAGE, triggering inflammation and redox imbalance. Elevated AGEs in uncontrolled diabetes increase RAGE expression, leading to apoptosis in pancreatic beta cells. The oxidative stress implicated in the progression of pancreatic beta cells dysfunction is due to the incapacitated pancreatic antioxidant defense system. The enhanced production of free radicals without an efficient cell defense mechanism leads to lipid peroxidation which may bring further cellular damage. The generation of damaging reactive oxygen species during oxidative stress affects the amyloidogenicity of islet amyloid polypeptide (IAPP) and leads to the formation and aggregation of toxic IAPP species. RAGE binds with these toxic IAPP intermediates and leads to the formation of amyloid plaque resulting in pancreatic beta cell demise.

To mitigate the harmful effects of diabetes mellitus, various oral antidiabetic medications are commercially available with distinct mechanisms of action. However, it's important to note that none of these medications have been found to effectively counteract the harmful accumulation of AGEs and RAGE expression within pancreatic beta cells. This study explored *Withania coagulans*, a medicinal plant with antioxidant and anti-inflammatory properties, as a potential treatment for AGEs-RAGE induced beta cells demise in the pancreas of streptozotocin (STZ-) induced chronic eight months diabetic male Wistar rats. *Withania coagulans* aqueous fruit extract (10mg/kg) administration for 40 days to chronic diabetic rats significantly detoxified the AGEs level, declined RAGE expression, regulated transcription factors MAFA and SOX9, normalized oxidative stress biomarkers [malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH)], and C-reactive protein (CRP). A significant increase in insulin-positive beta cells and improved pancreatic islets integrity was observed in the diabetic *Withania coagulans* treated group as compared to diabetic control. The novelty of our work is the regenerative effect of *Withania coagulans* in pancreatic beta cells mass, mediated through its antioxidant protective mechanism, AGEs detoxification, and modulation of the transcription factors implicated in beta cell survival and susceptibility to stress.

(P025) Targeting Heparan Sulfate Assembly for Drug Discovery in Rare Disease

Ryan Weiss[1,2]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602,* [2]*Department of Biochemistry and Molecular Biology, University of Georiga, Athens, GA 30602,*

Heparan sulfate proteoglycans (HSPGs) are ubiquitously expressed on all animal cells and in the extracellular matrix. Each HSPG consists of a core protein with one or more covalently attached linear heparan sulfate (HS) chains composed of alternating glucosamine and uronic acids that are heterogeneously *N*- and *O*-sulfated. These complex carbohydrates regulate many important biological processes including cell proliferation and development through their interaction with a large number of matrix proteins and growth factors. Dysregulation of HS assembly and catabolism has also been implicated in human diseases, including cancer and rare genetic disorders. Due to the key roles of HS in cell homeostasis and disease, there is much interest in targeting the assembly and function of HS as a therapeutic approach. To identify novel therapeutic agents and targets to tune HS assembly, we have generated reporter cell lines by fusing a small pro-luminescent peptide to the C-terminus of endogenous HS glycosyltransferase enzymes via CRISPR/Cas9 to enable sensitive monitoring of gene expression. We utilized these reporter lines in high-throughput screens of FDA-approved drugs to identify compounds that could modulate HS assembly. Multiple compounds were found to either increase or decrease HS gene expression, with enrichment of specific drug targets and pathways. Subsequently, the top hits from these screens were validated to inhibit or augment HS assembly in patient-derived cells for two rare genetic disorders, Sanfilippo Syndrome (MPS IIIA) and Multiple Hereditary Exostoses (MHE), respectively. Overall, these studies provide insight into the molecular mechanisms by which HS biogenesis is differentially regulated in cells and identify new therapeutic agents and targets to modulate HS assembly for disorders of glycosylation.

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(P026) Proteomic Changes to the Anterior Cerebral Artery and Adjacent Brain Tissue in Alzheimer's Disease

Margaret Downs[1], Xiaozhu Liu[2], Thor Stein[3], Yanhang Zhang[2,4,5], Joseph Zaia[1]

[1]*Department of Biochemistry and Cell Biology, Boston University Chobanian & Avedisian School of Medicine, Boston, MA,* [2]*Department of Mechanical Engineering, Boston University, Boston, MA,* [3]*Pathology and Laboratory Medicine, Boston University, Boston, MA,* [4]*Department of Biomedical Engineering, Boston University, Boston, MA,* [5]*Division of Materials Science and Engineering, Boston University, Boston, MA,*

Alzheimer's disease (AD) is the most common cause of dementia. In addition to the hallmark β-amyloid plaques and neurofibrillary tangles, AD is associated with structural vascular abnormalities and aberrant angiogenesis. Biomechanical changes including compliance reduction, artery stiffening, and structural degradation have been observed in the anterior cerebral artery (ACA). In recent studies, proteoglycans (PGs) have been implicated in maintenance of mechanical properties of arteries. As PGs are highly glycosylated molecules that have important structural and signaling roles in the extracellular matrix (ECM), we investigated the proteome and glycome of the ACA in pathological AD and control arteries as well as adjacent brain tissue. Proteins and glycosaminoglycans (GAGs) were digested from histological slides made from 1-mm thick artery rings from AD (n=6, Braak stages V-VI) and control (n=5, Braak stages 0-II), using our previously published on-slide digestion method. Brain tissue from AD (n=12) and control (n=12) was digested using the same method, selecting a 2-mm-diameter spot targeting grey matter. From arteries, proteomics analysis identified a total of ten proteins that were significantly differentially expressed (FDR< 0.05) from the AD group when compared to the controls, including four PGs and PG binding partners. Notably, the expression of basement membrane-specific heparan sulfate proteoglycan (PGBM) is decreased in AD, consistent with changes to vascular integrity.Using gene set enrichment analysis, several protein groups were found to be significantly differentially expressed in arteries. Growth factor binding, ECM structural constituent and protease binding were significantly enriched in AD. These groups consisted mainly of collagen family members, implying structural and signaling aberrations. Also, in the growth factor binding set HtrA serine peptidase 1 is present, which is known to target fibronectin and may also degrade ECM proteoglycans such as aggrecan and decorin. These findings also indicate PG abnormalities which may indicate compromised arterial wall integrity, leading to the altered biomechanics of the ACA. Further, in brain tissue, the mitochondrial matrix, mitochondrial inner membrane, and transport vesicle gene sets are significantly under-expressed in AD. This suggests a correlation between PG aberrations in arteries and dysfunction of the mitochondria, which has been linked to neurodegeneration. The alterations to ECM PGs and their interacting partners observed in cerebral arteries are indicative of ECM dysregulation in AD, which is consistent with the previously observed changes to arterial structure and integrity and correlates with malfunction of the mitochondria and vesicle trafficking in the brain. This is the first study to connect proteomic alterations to biomechanical changes in human cerebrovasculature in AD and to explore the relationship between arterial dysfunction and brain pathology.

(P027) Chondroitin Sulfate-driven Remodeling of Perineuronal Nets: Implications for Inflammation and Neurocircuit Dysfunction in Alzheimer's Disease

Cristiana Meuret[1], Angela Leung[2], Aarun Hendrickson[1], Jaden Le[1], Asmit Kumar[1], Ingrid Redford[2], Bao Anh Phan[2], Emily Quah[2], David Tovar[3], Ramon Sun[4], Carlos Campos[2], Jarrad Scarlett[2,5], Miklos Guttman[1], Kimberly Alonge[1,2]

[1]*Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA,* [2]*Department of Medicine, University of Washington, Seattle, WA, USA,* [3]*Department of Psychology, Vanderbilt University, Nashville, TN, USA,* [4]*Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, USA,* [5]*Department of Pediatric Gastroenterology and Hepatology, Seattle Children's Hospital, Seattle, WA, USA,*

Perineuronal nets (PNNs) are specialized extracellular matrix formations that envelop, and thereby regulate, neurons essential for memory and cognition. PNNs are comprised of chondroitin sulfate proteoglycans (CSPGs) with CS-glycosaminoglycan (CS-GAG) chain attachments. The CS disaccharide may be sulfated at the 2nd position of the glucuronic acid (GlcA) and the 4th and 6th positions of the *N*-acetylgalactosamine (GalNAc), forming combinations of non- (0S), mono- (4S and 6S), and di-(4S6S and 2S6S) sulfated CS isomers along the glycan polymer. Incorporation of differentially sulfated isomers into PNNs directly influences the stability of the underlying neurocircuitry. In Alzheimer's disease (AD), patients exhibit PNN destabilization, evident by the loss of histochemical PNN CS-glycan labeling, and we recently reported a significant increase in the 6S-CS isomer within the middle frontal gyrus of non-demented Braak III/IV patients that is further exacerbated in demented Braak V/VI patients. While overexpression of the 6S-CS isomer associates with altered neuronal activity, it also has been linked to inflammation-induced glial scarring. To elucidate the effects of 6S-CS overexpression on PNN matrix destabilization and neuroinflammation observed in AD patients, we unilaterally injected an adeno-associated virus 1 (AAV1) vector expressing the Chst3 (6S-sulfotransferase) (AAV1-CBA-[FLEXon]-mChst3-EGFP) in hippocampal GABAergic neurons using vgat-Cre mice. Fluorescent histochemical imaging revealed an increase in EGFP+ neurons on the ipsilateral hippocampus, indicative of AAV1-mChst3-EGFP expression, compared to the contralateral side that received the AAV1-CBA-[FLEXon]-tdTomato control. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of CS isomers isolated from hippocampal

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sections confirmed a 1.6±<0.1% (mean±SEM) increase in 6S-CS expression on the EGFP+ ipsilateral side, which was spatially verified by matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS). Overexpression of 6S-CS in GABAergic PNNs corresponded to a 39±12% reduction ($p<0.05$) in histochemical PNN labeling. Our preliminary data also showed overexpression of 6S-CS correlates with heightened neuroinflammatory responses, indicated by a 1.8±0.3-fold increase ($p<0.05$) in activated Iba+ microglia and a 2.3±0.4-fold increase ($p<0.05$) in activated GFAP+ astrocytes. We also observed the appearance of CS-GAG inclusion bodies on the EGFP+ ipsilateral hippocampus in association with the induction of neuroinflammation—a phenomenon we previously linked to traumatic brain injury. Summarily, overexpression of 6S-CS in GABAergic PNN matrices appears to drive destabilization of PNNs and glial neuroinflammation, revealing an interplay between PNN dynamics, CS-GAG sulfation patterns, and inflammation-driven pathologies underlying the AD pathogenesis.

(P028) Expression of Siglecs and Siglec ligands in the human cerebral cortex

Eila Maenpaa[1], Ronald L. Schnaar[1]

[1]*Department of Pharmacology & Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205 USA,*

Alzheimer's disease (AD) is the leading cause of dementia and is characterized by the buildup of misfolded proteins, phosphorylated tau intracellularly and amyloid β extracellularly. Genome wide association studies of AD susceptibility have implicated Siglec-3 also known as CD33 to increase risk of disease. Sialic-acid binding immunoglobulin-like lectins or better known as Siglecs, are transmembrane proteins that recognize sialoglycan ligands in their tissue environment and modulate immune responses. Siglec activation and immune inhibition occur when Siglecs on immune cell surfaces bind to their endogenous complementary sialoglycan ligand(s). The majority of Siglecs, including CD33 have an immunoreceptor tyrosine inhibitory motif that down-regulates the responses of the cells they're expressed on. Most Siglecs are expressed on subsets of immune cells, including microglia the resident phagocytic immune cell of the brain. Microglia mediate inflammatory responses and clear debris through phagocytosis. Microglia are beneficial when clearing debris and toxins, but can be detrimental when releasing proinflammatory mediators. Transcriptomic data reveled highest levels of Siglecs to be Siglec-8 and Siglec-10 on adult human microglia, with lower levels of Siglec-3, Siglec-7, Siglec-9, and Siglec-11 transcripts. To test if the transcriptomic data of the predominate Siglecs, as well as the Alzheimer's risk Siglec translates to protein expression, we obtained human brain tissue slices and co-stained them with α-IBA1, a mature microglia marker, and α-Siglec antibodies. We observed overlap of signal with α-IBA1 and α-Siglec-8, in multiple donor tissue slices as well as α-IBA1 and α-Siglec-3 in limited experiments. We also characterized the patterns of a common Siglec-3/Siglec-8 endogenous ligand, RPTPζ^{S3L}, in normal and AD cerebral cortex. These data support the hypothesis that human microglia express the protein Siglec-8 and Siglec-3 on their surface, where they can interact with the extracellular brain parenchymal ligand RPTPζ^{S3L}. This work was supported by the Cure Alzheimer's Fund.

(P029) Accumulation of Mannosylated Blood Proteins in Age-Related Vascular and Neurological Disease

Peter V. Aziz[1], Damien Restagno[1], Mayank Saraswat[1], Kathryn Smith[1], Jamey D. Marth[1]

[1]*Sanford Burnham Prebys Medical Discovery Institute,*

The vast majority of cell surface and secreted proteins are glycosylated with multi-antennary N-glycans linked to select asparagine residues. As nascent blood glycoproteins age in circulation, their glycan linkages are slowly hydrolyzed by the participation of circulating exo-glycosidases. This molecular aging process progressively exposes cryptic multivalent ligands of endocytic lectin receptors. In cases analyzed thus far, the rates of this glycan remodeling/aging among distinct glycoproteins are different and are inversely proportional to glycoprotein half-life and abundance. Alterations of this mechanism can change the rate of endocytic clearance, thereby rapidly altering the expression and function of various blood glycoproteins. Pathogens target this mechanism in multiple diseases including sepsis and colitis with impacts on pathogenesis and host outcome. To better understand this system and in particular the ligand repertoires of lectin receptors participating, we have optimized a methodology to isolate and subsequently identify physiological ligands of distinct mammalian lectin receptors. This methodology applies lectin (plant and animal) affinity chromatography in isolating non-denatured blood proteins followed by comparative mass spectrometry including lectin receptor-deficient mice. For example, we have recently identified over 100 mannosylated glycoprotein ligands of the Mrc1 lectin receptor accumulating in the blood of Mrc1-deficient mice. From meta-analyses of the data spanning biological processes and pathways that these glycoproteins participate in, we have successfully predicted the presence of previously undetected phenotypes. For example, Mrc1 deficiency resulted in the accumulation of mannosylated Renin and Angiotensin Converting Enzyme linked to elevations of systolic and diastolic blood pressure and cardiovascular fibrosis. The accumulation of mannosylated Myeloperoxidase tracked with elevated lipid oxidation, vascular inflammation, and tissue leukocyte infiltration. Moreover, absence of Mrc1 resulted in the detection of mannosylated neurological proteins in the blood. Neurological proteins are not detectable in healthy mammals by approaches including mass spectrometry. Dysfunction of the blood brain barrier was

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observed with various regions of the brain exhibiting inflammation and neuronal death. These findings support the ongoing identification of physiological ligands of mammalian lectin receptors as a means to understand control of blood proteostasis in the pathogenesis of disease. We have begun studies to analyze mannosylated glycoprotein levels in the blood of healthy humans of multiple ages and human patients diagnosed with inflammatory diseases including sepsis.

(P030) O-GlcNAcylation dependent transcriptomic changes in hyperglycemia-primed atrial fibrillation

Emily Juzwiak[2], Jonathan Granger[1], Olurotimi Mesubi[1]

[1]Department of Medicine, The Johns Hopkins University School of medicine, Baltimore, MD, USA, [2]Department of Cellular and Molecular Medicine, The Johns Hopkins University School of medicine, Baltimore, MD, USA,

Rationale: Excessive O-GlcNAcylation (O-GlcNAc) is a hallmark of diabetes mellitus (DM) which is a major risk factor for atrial fibrillation (AF), the most common clinical arrhythmia. O-GlcNAc is dynamically regulated by 2 enzymes - O-GlcNAc transferase (OGT) which catalyzes the addition of UDP-GlcNAc to proteins and O-GlcNAcase (OGA) which hydrolyses the removal of UDP-GlcNAc from proteins. The mechanism by which O-GlcNAc promotes hyperglycemia-primed AF is unknown.
Objective: To determine the mechanistic role of O-GlcNAc in hyperglycemia-primed AF via transcriptomic profiling of murine heart samples.
Methods: We used a type 2 DM (T2D) mouse model (high fat diet (60% Kcal fat) + low dose streptozocin) and transgenic mice with cardiac specific OGA overexpression (OGA-TG). We compared transcriptomes by RNA sequencing in hearts from non-diabetic and T2D OGA-TG and OGA-WT mice. To assess genes potentially affected by O-GlcNAc in this model, gene and transcript expression levels (FKPM [fragments per kilobase of transcript per million] values) were compared among hearts from OGA-WT non-DM and T2D, OGA-TG non-DM, and T2D mice.
Results: In WT T2D mice, increased myocardial O-GlcNAc is associated with increased AF susceptibility compared with non-diabetic littermates. OGA-TG T2D mice are protected from increased AF susceptibility. We found 2484 genes significantly changed in OGA-WT vs OGA-TG, 999 genes in the OGA-WT vs OGA-WT T2D, 92 genes in OGA-WT vs OGA-TG T2D, and 865 genes in the OGA TG vs OGA-TG T2D hearts. Pathway analysis of the top canonical and biological functions identified enrichment of mitochondrial pathways – gene expression, translation, respiratory complex assembly in the OGA-TG T2D hearts vs OGA-WT T2D hearts. Further we found enrichment of lipid and fatty acid oxidation and metabolism in OGA-TG T2D vs OGA-TG non-DM hearts. Examples of upregulated genes in the OGA-TG T2D hearts include Prune2, Ndrg4, and Armc2 while examples of downregulated genes in the OGA-TG T2D hearts include Rab11b, Slc47a1, Eif4e3 and Lmo1.
Conclusions: Myocardial transgenic overexpression of OGA protects from hyperglycemia-primed AF and transcriptomic profiling identified enrichment of mitochondrial, lipid and fatty acid oxidation and metabolism as the most prominent pathways differentially affected. Further studies are needed to understand the role of O-GlcNAc signaling in AF.

(P031) Galectin-4 antimicrobial activity primarily occurs through its C-terminal domain

Hau-Ming Jan[1,*], Shang-Chuen Wu[1,*], Carter J. Stowell[1], Mary L. Vallecillo-Zúniga[1], Anu Paul[1], Kashyap R. Patel[1], Diyoly Ayona[1], Ryan Philip Jajosky[1], Samata P. Varadkar[1], Hirotomo Nakahara[1], Rita Chan[2], Devika Bhave[2], William J. Lane[1], Melissa Yeung[3], Marie Hollenhorst[3], Seth Rakoff-Nahoum[2], Richard D. Cummings[4], Connie M. Arthur[1], Sean R. Stowell[1]

[1]Joint Program in Transfusion Medicine, Brigham and Women's Hospital, [2]Infectious Disease Division, Boston Children's Hospital, [3]Department of Medicine, Brigham and Women's Hospital, [4]Harvard Glycomics Center, Beth Israel Deaconess Medical Center, Harvard Medical School

While immune tolerance has evolved to minimize reactivity with self-components, it results in a deficiency in the adaptive immune response when microbes camouflage themselves with self-like antigens. This becomes especially evident in the case of carbohydrate-based blood group antigens, where microbes have the ability to envelope themselves with blood group structures resembling those found on human cells. In this study, we demonstrate that the innate immune lectin, galectin-4 (Gal-4), exhibits strain-specific binding and killing behavior towards microbes that display blood group-like antigens. Examination of binding preferences using a combination of microarrays populated with ABO(H) glycans and a variety of microbial strains, including those that express blood group-like antigens, demonstrated that Gal-4 binds mammalian and microbial antigens that have features of blood group and mammalian-like structures. Although Gal-4 was thought to exist as a monomer that achieves functional bivalency through its two linked carbohydrate recognition domains (CRDs), our data demonstrate that Gal-4 forms dimers and that differences in the intrinsic ability of each domain to dimerize likely influences binding affinity. While each Gal-4 domain exhibited blood group binding activity, the C-terminal domain (Gal-4C) exhibited dimeric properties, while the N-terminal domain (Gal-4N) failed to similarly display dimeric activity. Gal-4C not only exhibited the ability to dimerize, but also possessed higher affinity toward ABO(H) blood group antigens and microbes expressing glycans with blood group-like features. Furthermore, when compared to Gal-4N, Gal-4C exhibited more potent antimicrobial activity. Even in the context of the full-length protein, where Gal-4N is functionally bivalent by virtue of Gal-4C dimerization, Gal-4C continued to display higher

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antimicrobial activity. These findings illustrate the dimeric nature of Gal-4 and reveal that its antimicrobial activity is primarily mediated by its C-terminal domain. These data also offer valuable insights into the critical attributes of Gal-4 responsible for its innate immune response against molecular mimicry.

(P032) N-glycoproteomics reveals distinct glycosylation alterations in patients with ALG1-CDG

Rohit Budhraja[1], Neha Joshi[1,2], Tamas Kozicz[1,3], Eva Morava[1,3], Akhilesh Pandey[1,2,3]

[1]*Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA,* [2]*Manipal Academy of Higher Education (MAHE), Manipal, India,* [3]*Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA,* [4]*Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA*

Congenital disorders of glycosylation (CDG) are a group of rare genetic disorders characterized by abnormalities in the process of glycosylation. CDG are caused by pathogenic variants that affect the enzymes involved in glycosylation, leading to dysfunctional or incomplete glycan structures. Asparagine-linked glycosylation 1 protein is encoded by the *ALG1* gene which is a β-1,4-mannosyltransferase. The ALG1 enzyme catalyzes the first mannosylation step in the biosynthesis of dolichol-linked oligosaccharide. Defect in *ALG1* causes a rare autosomal recessive disorder designated ALG1-CDG. To date, more than 80 patients have been documented with this multisystem disorder, which encompasses complications affecting the central nervous system as well. It is important to note that transferring glycosylation is not a dependable diagnostic method for these patients. Regrettably, as of now, there is no known therapy available for ALG1-CDG. However, despite the essential role of ALG1 in glycosylation pathway, the exact consequences of ALG1 deficiency on global cellular protein abundance and their glycosylation have not yet been investigated. Thus, we performed a multiplexed mass spectrometry-based quantitative proteomics and glycoproteomic study using tandem mass tags (TMT) in ALG1 deficient patient fibroblasts. This global quantitative mass spectrometric analysis defined a proteomic signature of differential expression across patient and control fibroblasts. Proteins that showed significant differential expression included pleckstrin, four and a half LIM domains protein 1, platelet-derived growth factor D, thymosin beta-4, hyaluronan and proteoglycan link protein 1, sorbin and SH3 domain-containing protein 1 and ALG1. We also observed a significant decrease in the expression of mitochondria- and increase in autophagy- related proteins which indicates the cellular stress. We detected a total of 3,914 N-glycopeptides derived from 485 glycoproteins possessing 249 unique glycan compositions. Notably, patient fibroblasts exhibited a substantial reduction in high-mannose and complex/hybrid glycopeptides originating from multiple proteins. Moreover, we observed a marked increase in several novel oligosaccharides in patients, including the tetrasaccharide (NeuGc-Gal-GlcNAc2), trisaccharide (Gal-GlcNAc2), and disaccharide (GlcNAc2). These oligosaccharide structures were found on several important cellular proteins including lysosomal-associated membrane protein 1, CD44, and integrin subunits. The accumulation of such smaller oligosaccharides can be attributed to the fact that ALG1 plays a critical role in the early stages of glycosylation biosynthesis. Overall, our findings not only offer short oligosaccharide-bearing peptides as potentially novel biomarkers but also shed light on new molecular and system-level insights into understanding ALG1-CDG.

(P033) A complement C4-derived glycopeptide as a novel biomarker for PMM2-CDG

Kishore Garapati[1,2,3,†], Rohit Budhraja[1,†], Mayank Saraswat[1], Jinyong Kim[1], Neha Joshi[1,2,3], Gunveen S. Sachdeva[1,3], Anu Jain[1], Anna N. Ligezka[4], Silvia Radenkovic[4], Madan Gopal Ramarajan[1,2,3], Savita Udainiya[1,2,3], Kimiyo Raymond[5], Miao He[6], Christina Lam[7,8], Austin Larson[9], Andrew C. Edmondson[10], Kyriakie Sarafoglou[11,12], Nicholas B. Larson[13], Hudson H. Freeze[14], Matthew J. Schultz[5], Tamas Kozicz[4,5,15,16], Eva Morava[4,5,15,16,*], Akhilesh Pandey[1,16,*]

[1]*Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA,* [2]*Institute of Bioinformatics, International Technology Park, Bangalore, Karnataka, India,* [3]*Manipal Academy of Higher Education (MAHE), Manipal, Karnataka, India,* [4]*Department of Clinical Genomics, Mayo Clinic, Rochester, Minnesota, USA,* [5]*Biochemical Genetics Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA,* [6]*Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA,* [7]*Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, Washington, USA,* [8]*Division of Genetic Medicine, Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington, USA,* [9]*Colorado Children's Hospital, Denver, Colorado, USA,* [10]*Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA,* [11]*Department of Pediatrics, Division of Pediatric Endocrinology, University of Minnesota Medical School, Minneapolis, Minnesota, USA,* [12]*Department of Experimental and Clinical Pharmacology, University of Minnesota School of Pharmacy, Minneapolis, Minnesota, USA,* [13]*Department of Quantitative Health Sciences, Mayo Clinic, Rochester, Minnesota, USA,* [14]*Sanford Children's Health Research Center, Sanford-Burnham-Prebys Medical Discovery Institute, La Jolla, California, USA,* [15]*Department of Anatomy, University of Pecs Medical School, Pecs, Hungary,* [16]*Center for Individualized Medicine, Mayo Clinic, Rochester, Minnesota, USA,*

*Corresponding authors

†These authors contributed equally

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The most common congenital disorder of glycosylation (CDG), PMM2-CDG, is caused by deficiency of the cytosolic enzyme phosphomannomutase. Pathogenic variants in the encoding gene, *PMM2*, lead to the reduced availability of glycosylation precursors like mannose-1-phosphate. Hypoglycosylation is a hallmark of this type I CDG where N-linked glycosylation sites of proteins are unoccupied. Hypoglycosylation of transferrin is quantified by mass spectrometry-based measurements of the intact protein. Clinical testing for carbohydrate-deficient transferrin (CDT), along with enzyme activity and genetic testing, are used for the diagnosis of PMM2-CDG. However, CDT levels in many patients are normal, and can also normalize with age, reducing its reliability for screening for PMM2-CDG. Several recent reports indicate that N-glycans in sera from PMM2-CDG patients show alterations in glycan structures. Notably, although recent advances in mass spectrometry-based glycopeptide studies have made possible the study of site-specific glycosylation changes of proteins, such alterations have not been reported in PMM2-CDG patient sera. To address this gap, we used mass spectrometry-based glycoproteomics through a multi-step approach to analyze sera from 72 individuals (35 with PMM2-CDG) for discovery and validation of glycopeptide alterations. First, we identified signature changes among 3,342 glycopeptides from 284 circulating glycoproteins in a comprehensive TMT-based discovery experiment. Specific glycosylation sites showed remarkable changes with a decrease in mature glycopeptides and a shift towards truncated glycopeptides. We observed a significant increase in the levels of a high mannose glycopeptide from complement C4 and three truncated glycopeptides derived from alpha-1-acid glycoproteins 1 and 2 and coagulation factor XII in PMM2-CDG patients, as well as of glycopeptides from haptoglobin with glycosylation on only one of two possible sites (but not both) of N-glycosylation. These changes were tested by profiling an additional set of samples using an alternate streamlined method. We identified a glycopeptide from complement C4 carrying the glycan $Man_5GlcNAc_2$ in all affected individuals but not in controls, and confirmed it using targeted analysis of blinded samples. We believe that this C4-derived $Man_5GlcNAc_2$ glycopeptide could serve as a novel biomarker for PMM2-CDG. Additionally, these changes in the heterogeneity of site-specific glycosylation in PMM2-CDG improve our understanding of this disorder and how glycosylation is affected in type I CDGs.

(P034) Deglycosylation of perineuronal nets in mouse models of tauopathy

Kimberly M. Alonge[1,2], Brian Foresi[3], Shannon Hu[4], Aarun Hendrickson[1], Ingrid K. Redford[2], Asmit Kumar[1], Emily Quah[2], Bao Anh Phan[2], Tammy Doan[5], Cassidy Noonan[2,6], Nzinga E. Hendricks[6], Jeanna M. Wheeler[6], Brian C. Kraemer[2,6], Aric F. Logsdon[2,6]

[1]*Department of Medicinal Chemistry, University of Washington, Seattle, WA,* [2]*Department of Medicine, University of Washington, Seattle, WA,* [3]*College of Medicine, Northeast Ohio Medical University, Rootstown, OH,* [4]*Vollum Institute, Oregon Health & Science University, Portland, OR,* [5]*Department of Biochemistry and Molecular Medicine, University of Southern California, Los Angeles, CA,* [6]*Geriatric Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, WA,*

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by clinical symptoms of memory and cognitive loss. The hallmark neuropathological criteria for AD diagnosis include the accumulation of extracellular beta amyloid ($A\beta$), but current therapies targeting $A\beta$ have limited clinical efficacy and life-threatening side effects. These limitations highlight the need for alternative treatments targeting other AD neuropathologies, including intracellular hyperphosphorylated tau (pTau).

The brain's extracellular matrix, particularly perineuronal nets (PNNs), plays a crucial role in brain functioning and neurocircuit stability, and reorganization of these unique matrices have been associated with pTau accumulation in human AD brain tissue. Here, we investigated PNN integrity and chondroitin sulfate proteoglycan (CSPG) compositional changes in transgenic mouse models expressing tauopathy-related AD pathology, PS19 and Tg2652 mice. We show that 9-month PS19 mice exhibit loss of hippocampal PNN CS glycans using *Wisteria floribunda* agglutinin (WFA) ($p<0.0001$), but not the underlying CSPG core proteins (aggrecan ($p=0.63$); Cat-315 ($p=0.75$), in association with pTau accumulation ($p<0.0001$), microgliosis ($p<0.0001$), and astrogliosis ($p<0.0001$) (n=10 WT, 5M/5F; n=10 PS19, 5M/5F). These findings suggest PS19 exhibit PNN deglycosylation but not loss of the PNN matrix itself. Further investigation of 9-month PS19 CS glycan composition shows shifts in in their hippocampal CS sulfation patterns to favor expression of the regenerative 2S6S-CS isomer ($p=0.02$), which also strongly associated with neurodegeneration within the dentate gyrus ($p=0.01$). Considering our recent report showing upregulation of the 2S6S-CS isomer in demented Braak V/VI human patient brain tissue, we predict that the increased expression of 2S6S-CS in PS19 mice might occur only after the appearance of pTau. To test this hypothesis, we investigated a second mouse model of tauopathy, Tg2652 mice, which exhibit robust pTau accumulation in the absence of gliosis. Surprisingly, we found Tg2652 mice exhibited stable hippocampal PNN structures and normal CS glycan isomer composition despite robust pTau accumulation, suggesting a critical role between neuronal PNN stability and activation of neighboring glial cells in the PS19 model. Overall, our findings provide insights into the relationship between PNN CS glycans, pTau pathology, gliosis, and neurodegeneration in mouse models of tauopathy. The 2S6S-CS isomer is a critical variant shown to exhibit neuroregenerative properties, including increasing neurite bearing neurons, process length, and outgrowth in hippocampal cell cultures, and we now predict that the increase in 2S6S-CS isomer in demented Braak V/VI patients and 9m-PS19 mice may represent a biological response to the pTau-associated neurodegeneration, thus highlighting this isomer as a potential novel therapeutic targets for AD treatment.

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(P035) Revealing the extent of disease progression through glycomic profiling

Yasmine Bouchibti[1]

[1]UC Davis,

Glycosylation is a post translational modification of proteins adding various sugar structures, glycans, which play a role in structure and function. The overall glycomic profile has biological relevance as it offers insight on the glycosylation pathway and is used to identify biomarkers for disease. This work aims to identify possible biomarkers in different disease models through MS based analytical methods analyzing the glycocalyx. With this approach, native N and O-glycans are isolated from various biological samples ranging from glioblastoma xenografts to vaginal swabs. Glycomic analysis is conducted on a nanoLC-Chip-QTOF MS system allowing for isomeric separation and identification of unique glycan structures through in-house built libraries from retention time and fragmentation data. In glioblastoma studies with xenografts glycomic profiling of the tumor initiating cells confirmed the correlation between tumorigenic activity and $\alpha(2,6)$ sialylation of glycans initiated by the ST6Gal1 glycotransferase. The isomeric separation capability of the method illustrated the distinct differences in the glycome where the sialylation enzyme was knocked down. Under bacterial vaginosis studies, the extent of the biofilm disruption by the bacterial communities is revealed by the degree of de-sialylation on the O-glycosylated mucins. Increased presence of these sialic acid cleaved structures across healthy and diseased state is evident from the glycomic analysis of vaginal swabs. Expanding the work to other disease models will elucidate other glycosylation associated patterns that are seen with the progression of disease.

(P036) Mouse model of PMM2-CDG demonstrates neurodevelopmental origin of brain pathology

Andrew Edmondson[1,2], Cadmus Cai[2], Emily Shiplett[1], Miao He[1], Silvia Radenkovic[3], Eva Morava[3], Zhaolan Zhou[2]

[1]Children's Hospital of Philadelphia, [2]University of Pennsylvania, [3]Mayo Clinic,

Congenital disorders of glycosylation (CDG) are a group of neurogenetic disorders that disrupt
cellular glycosylation machinery. The majority of CDG patients have biallelic mutations in *PMM2*. Affected PMM2-CDG individuals suffer from multi-systemic symptoms and experience prominent neurological deficits, the most consistent being cerebellar hypoplasia with resulting ataxia. Mouse models of PMM2-CDG have been paradoxically problematic due to either excessive embryonic lethality or an apparent lack of phenotype. Even a recently described compound heterozygous knock-in mouse model with the most common patient genotype has excessive embryonic lethality without apparent brain pathology. To generate a neural knock-out model of *Pmm2* while avoiding excessive embryonic lethality, we utilized a mouse with conditional *Pmm2* allele and brain-specific Cre lines. Western analysis, PMM enzyme activity, and glycomic analysis confirmed loss of PMM2. Different brain specific Cre lines resulted in drastically different phenotypes, with lines expressing Cre in late embryonic/early postnatal timepoints and targeting post-mitotic neurons resulting in mice without a neurologic phenotype assayed across numerous behavioral and learning domains. However, lines expressing Cre during mid-embryonic stages and targeting neural precursors resulted in mice recapitulating key aspects of PMM2-CDG neurological involvement, including ataxia and early mortality. Metabolic profiling in affected mice localized the largest metabolic disruptions to cerebellum. Ongoing studies seek to identify key cell-type specific disruptions due to PMM2 loss in the developing mouse brain and to identify glycoproteomic disruptions. This conditional *Pmm2* mouse allele is a powerful tool to enable study of organ-specific biological functions of N-glycans and to study pathogenic mechanisms of PMM2-CDG while avoiding embryonic lethality.

(P037) Metabolic clogging of mannose triggers dNTP loss and genomic instability in human cancer cells

Yu Mizote[1], Takehiro Suzuki[2], Akiyoshi Hirayama[3], Mikako Nishida[4], Toru Hiratsuka[1], Yusuke Imagawa[1], Junko Murai[3], Hudson H. Freeze[5], Eiji Miyoshi[6], Shigeki Higashiyama[1], Heiichiro Udono[4], Naoshi Dohmae[2], Hideaki Tahara[1], Naoyuki Taniguchi[1], Yoichiro Harada[1]

[1]Osaka International Cancer Institute, [2]RIKEN, [3]Keio University, [4]Okayama University, [5]Sanford Burnham Prebys Medical Discovery Institute, [6]Osaka University,

Mannose has anti-cancer activity that inhibits cell proliferation and enhances the efficacy of chemotherapy. How mannose exerts its anti-cancer activity, however, remains poorly understood. Here, using genetically engineered human cancer cells that permit the precise control of mannose metabolic flux, we demonstrate that the large influx of mannose exceeding its metabolic capacity induced metabolic remodeling, leading to the generation of slow-cycling cells with limited deoxyribonucleoside triphosphates (dNTPs). This metabolic remodeling impaired dormant origin firing required to rescue stalled forks by cisplatin, thus exacerbating replication stress. Importantly, pharmacological inhibition of *de novo* dNTP biosynthesis was sufficient to

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retard cell cycle progression, sensitize cells to cisplatin, and inhibit dormant origin firing, suggesting dNTP loss-induced genomic instability as a central mechanism for the anti-cancer activity of mannose.

(P038) Elucidating the O-glycan structure of the novel periodontal health-associated bacterium Tannerella serpentiformis

Stephanie Walcher[1], Fiona Hager-Mair[1], Johannes Stadlmann[1], Hanspeter Kählig[2], Christina Schäffer[1]

[1]*Institute of Biochemistry, Department of Chemistry, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria,* [2]*Department of Chemistry, University of Vienna, Währinger Straße 38, 1090 Vienna, Austria,*

Tannerella serpentiformis, the closest phylogenetic relative of the periodontal pathogen *Tannerella forsythia,* was recently discovered to play a remarkably health-associated role in the oral biofilm community. For instance, *T. serpentiformis* shows a decreased invasiveness of host cells compared to *T. forsythia*. [1]. Furthermore, the striking difference in biofilm behavior between the two *Tannerella* species is accompanied by a notable lack of nonulosonic acid biosynthesis genes in the *T. serpentiformis* glycosylation gene cluster. Nonulosonic acids are well-known for their pivotal role in bacterial pathogenicity and it has been shown that a modified nonulosonic acid is characteristic of the S-layer *O*-glycan of pathogenic *T. forsythia* strains, where the S-layer is a virulence factor [2, 3]. We hypothesize that differences in the glycans displayed on the *T. serpentiformis* cell surface contribute to the bacterium's dissimilar pathogenicity potential and lifestyle.

In this study, we elucidated the structure of the *T. serpentiformis* S-layer *O*-glycan by NMR and MS analysis and found that it is present in two main glycoforms, which differ only slightly in linkages and composition. We identified seven different monosaccharides that make up a branched deka- or undekasaccharide and carry (non-)carbohydrate modifications. There are four to five fucoses present in each glycoforms, which are O-methylated and in one case modified with an NH_2 group, which has been described only once in the literature, so far. Importantly, no nonulosonic acid is present in the *O*-glycan and a hexose serves as linking sugar to the protein. Moreover, we could identify over 200 individual heavily glycosylated proteins in *T. serpentiformis*, including the bacterium's abundant surface layer glycoproteins. Beyond that, our data support the phylum-wide postulated *O*-glycosylation amino acid motif [4] based on bioinformatics analysis of MS data against a background of non-glycosylated sequences.

This work was supported by the Austrian Science Fund FWF, project P33619-B22 (to C.S), and the doctoral program W1124 (BioToP).

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(P039) Using System Biology Approaches to Understand the O-GlcNAc Rheostat

Chad Slawson[1], Samuel Boyd[2], Dakota Robarts[3], Khue Nguyen[1], Manasi Kotulkar[3], Maite Villar[1], Antonio Artigues[1], Ibtihal Alghusen[1], Jeffrey Thompson[2], Wagner B. Dias[4], Udayan Apte[3]

[1]*Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA,* [2]*Department of Biostatistics and Data Science, University of Kansas Medical Center, Kansas City, KS, USA,* [3]*Department of Pharmacology and Toxicolgy, University of Kansas Medical Center, Kansas City, KS, USA,* [4]*Laboratório de Glicobiologia Estrutural e Funcional, Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, 21941-902, Brazil*

Like all post-translational modifications, O-GlcNAc regulates protein function, stability, localization, or interactions; thus, O-GlcNAcylation regulates a myriad of cellular functions. However, determining how O-GlcNAc controls cell function is not a trivial task. Importantly, O-GlcNAc is processed by a single O-GlcNAc transferase (OGT), which adds the modification, and a single O-GlcNAcase (OGA), which removes the modification, and allows OGT and OGA to process the modification like a rheostat. Numerous studies demonstrate that cells oscillate O-GlcNAc levels to modify signaling pathways in response to environmental changes. Here, having just one enzyme to add or remove the modification allows the cell to harmonize all cellular function with fluctuating nutrient or growth signals. This single control point coordinates cellular processes, but O-GlcNAc changes could have subtle and seemingly minor effects at the level of a single protein; although across the cell, the cumulative changes would be profound. For example, breaking the rheostat by knocking out either of the O-GlcNAc

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processing enzymes OGT and OGA is lethal in growing and dividing cells. Thus, the O-GlcNAc rheostat is the perfect way for multicellular eukaryotes to link the cell cycle to nutrient availability. Since, O-GlcNAc does everything, everywhere, all at once; then, understanding the biological significance of this modification is difficult. Therefore, we are developing a systems level view of O-GlcNAcylation to understand how the modification controls cell function. First, we combined Multi-omics with novel tissue specific OGT/OGA knockout animals to understand how manipulating the O-GlcNAc enzymes affects cellular processes. Second, we developed a bioinformatics tool (AMEND) to align and process changes in Multi-omic data, and third we are improving O-GlcNAc proteomic methods to couple O-GlcNAc site-mapping with quantitation. When these methods were tested in OGT/OGA knockout liver or liver from mice treated with an OGA inhibitor for 1 or 2 weeks, we found substantial changes in metabolism and cell cycle regulation. Bioinformatics predicted changes in cell cycle progression which was supported by previous findings as well large increases in liver aneuploidy in OGT KO animals. Additionally, we found substantial changes in the Hippo signaling pathway. Together, a combinatorial Multi-omics approach with advancements in bioinformatics and animal models predicts at the cellular level how O-GlcNAc affects a cells function. We argue our systems biology approach will unlock new mechanisms of the O-GlcNAc rheostat and allow for a broader understanding of O-GlcNAcylation.

(P040) A new Glycan-peptide crosslinking LC-MS method with AI-protein folding yield new interaction networks in cell membrane

Michael Russelle Alvarez[1,†], Siyu Chen[1,†], Yixuan Xie[1], Ying Sheng[1], Yasmine Bouchibti[1], Vincent Chang[1], Carlito B. Lebrilla[1,2]

[1]*Department of Chemistry, University of California, Davis, CA, USA,* [2]*Department of Biochemistry, University of California, Davis, CA, USA,*

[†]Contributed equally

Glycosylation is an important post-translational modification of proteins that aids in facilitating their biological functions. Due to the vast diversity and heterogeneity of N-glycan structures, it is currently unknown how each specific structure contributes to its biological function. Crosslinking mass spectrometry (XL-MS) experiments have previously been used to examine protein-protein interactions, but these methods focused primarily on the interactions of the proteins while ignoring the contributions of glycosylation. This report utilized a newly developed enrichable glycan-peptide crosslinker to probe the role of fucosylation and structural heterogeneity in the protein-protein interaction landscapes of the cell surface. Using our glycan XL-MS method, we constructed protein-protein networks of prostate cells (PNT2) that are interconnected by specific glycan structures with varying amounts of fucosylation. Interestingly, we observed that glycans with a higher number of fucose residues connected a higher degree of proteins compared to glycans with a lower number of fucose. We performed AI-enabled molecular modeling and dynamics simulations to provide an explanation and found that fucosylation, especially core-fucosylation, drastically alters the glycan conformation in the glycoprotein. Furthermore, we found that each specific glycan structure connects a unique set of proteins and pathways, thereby demonstrating that glycan heterogeneity allows for proteins to moderate various and specific functions. Using molecular modeling, we constructed multimeric protein complexes of integrins beta-1 and alpha-V, with cell adhesion molecule ALCAM, showing the interactions of the glycans in the structures.

(P041) FUT10 and FUT11 are novel protein O-fucosyltransferases that modify EMI domains

Huilin Hao[1], Youxi Yuan[1], Atsuko Ito[1], Benjamin M. Eberand[2], Michelle Cielesh[2], Mark Larance[2], Robert S. Haltiwanger[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30605,* [2]*Charles Perkins Centre, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia,*

Fucosylated glycan structures are commonly found in mammalian cells and play vital roles in diverse biological events. Among the 13 identified fucosyltransferases (FUTs) in human, only Protein *O*-fucosyltransferases (POFUTs) 1 and 2 directly transfer fucose to protein serine or threonine residues via *O*-linkage. Both POFUT1 and POFUT2 require the recognition of specific consensus sequences within certain cysteine-rich domains: Epidermal Growth Factor-like (EGF) repeats and Thrombospondin Type 1 Repeats (TSRs), respectively. *O*-fucose glycans modulate protein function through direct intermolecular interactions with binding partners, regulating processes such as Notch-ligand binding. They also form intramolecular interactions that stabilize EGF repeats and TSRs, thereby aiding in proper folding. In addition to EGF repeats and TSRs, a recent platelet proteome study identified a novel *O*-fucose site on the elastin microfibril interface (EMI) domain of Multimerin-1 (MMRN1), a major platelet protein that supports platelet adhesion and thrombus formation. We found that neither POFUT1, nor POFUT2, was responsible for this modification, suggesting that a novel POFUT may exist for modifying protein EMI domains. In Alphafold2 screens of binding structures involving the EMI domain, the closely related FUT10 and FUT11 enzymes demonstrated significant EMI binding that was not observed with other FUTs. Robust *O*-fucosylation activity was observed in our *in vitro* assays using purified, recombinant FUT10/11 and EMI substrates combined with mass spectrometric analysis. Knocking out of either *FUT10,* or *FUT11,* in HEK293T cells substantially decreased the stoichiometry of *O*-fucosylated peptides of EMI. These findings imply that

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FUT10 and FUT11 function as POFUTs for EMI domains. EMI *O*-fucosylation remained unaffected in knockout of the Golgi GDP-fucose transporter, *SLC35C1*, in HEK293T cells compared to WT cells, which combined with subcellular localisation data demonstrate FUT10/11 function in the ER. Much like POFUT1 and POFUT2, both FUT10 and FUT11 rely on properly folded EMI structures for efficient modification. Notably, mutating the O-fucose sites of the MMRN1 EMI domain led to a ~50% reduction in MMRN1 secretion. These findings suggest that akin to POFUT1 and POFUT2, FUT10 and FUT11 participate in a non-canonical ER quality control pathway for EMI domains. This work was supported by NIH grant R35GM148433.

(P042) Probing the glycosylation landscape of the human cornea

Rafael Martinez-Carrasco[1], Pablo Argüeso[1]

[1]*Department of Ophthalmology, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA,*

The surface of every cell is covered with a meshwork of carbohydrate-enriched structures known as the glycocalyx. This glycocalyx displays variations in both its composition and functions across different cell types and tissues, enabling it to fulfill specific roles based on the location of the cell within the body. The advent of high-throughput sequencing technologies has facilitated the profiling of glycosylation genes in individual cells within complex biological systems. However, the significance of these gene signatures in shaping the composition of the glycocalyx remains uncertain. In this study, we used lectin microarrays to characterize the composition of cell surface glycans in the human cornea and determine its relationship to single-nucleus transcriptomic data. Our results unveil a series of distinctive cell surface glycan signatures that are exclusive to different cell types of the human cornea. These signatures exhibit a certain degree of correlation with the transcriptional expression of glycosylation genes. Epithelial cells showcase a stronger binding affinity to O-glycan lectins (BPA, DISCOIDIN I), a trait linked to their increased expression of polypeptide GalNAc transferases (*GALNT3, 7, 12, 13, 17, 18*) and *C1GALT1*. Conversely, stromal and endothelial cells exhibit an elevated level of core fucose, as indicated by the differential expression of *FUT8* and the increased surface protein binding to PSA. Both transcriptomic data and lectin array analysis suggest a limited presence of sialic acid in stromal cells, while epithelial and endothelial cells distinctly feature α2-6 (PSL1A) and α2-3 (MAA) sialic acid, respectively. These results could provide insights into the specialized function of individual cell types within the cornea and foster the identification of novel cell-specific biomarkers.

Acknowledgments: This work was supported by the National Institutes of Health NEI Grant R01EY026147, Massachusetts Lions Eye Research Fund, Inc.; Research to Prevent Blindness, Inc., and the New England Corneal Transplant Research Fund.

(P043) Identification of Putative Glycosyltransferases from Environmentally Isolated Bacteria Involved in Glycosphingolipids Biosynthesis

Tahir Ali[1], Paul D. Boudreau[1]

[1]*Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, MS, USA,*

As exemplified through the COVID-19 pandemic, vaccines are a powerful tool to reduce the prevalence of disease and the risk of death associated with the infectious diseases. A typical vaccine is composed of an antigen and an adjuvant to enhance the immune response. Natural products, such as glycosphingolipids, offer potential as new adjuvants for vaccines. Bacterial glycosphingolipids (GSLs), including α-galactosylceramide and α-glucuronosylceramide, have been identified as ligands for the CD1d antigen receptor of the immune system. Although the structure and biological activities of GSLs are well documented, there is limited information on the glycosyltransferases, such as glucuronosyltransferase (GlcA-T), responsible for their biosynthesis in bacteria. We set out to identify and characterize the glycosyltransferases involved in the biosynthesis of GSLs from two strains of environmentally isolated bacteria belonging to the *Novosphingobium* genus. First, using nanopore sequencing of high molecular weight DNA extracts of two of our *Novosphingobium* strains, we generated draft genomes. Annotations of these genomes with RAST were then interrogated for putative glycosyltransferases. Which we assembled into a sequence similarity network using the EFI-Enzyme Similarity Tool before visualizing through the Cytoscape software platform. This analysis included protein sequences from our two *Novosphingobium* strains, other bacteria belonging to the order Sphingomonadales, reference sequences for the three known bacterial sphingolipid glycosyltransferases, and two bifunctional monoglucosyl/glucuronosyl diacylglycerol synthases. Interestingly, the network showed clustering of multiple genes from our strains to the known GlcA-T proteins. Some of the identified hits are proteins of smaller size, which could be attributed to sequencing errors. To rectify these errors, we amplified these genes by PCR and subjected them to Sanger sequencing. By including the Sanger-corrected sequences, we were able to construct a SSN network that more accurately represents the genes within this cluster. This network showed two homologs of the GlcA-T enzyme in our strains. We hypothesize the two homologs are engaged in a different sugar chemistry pathway, but they may also employ dissimilar acceptor substrates. In future research, we plan to examine the roles of these two proteins in our strains through knockout experiments, comparing the lipidomic profiles of the knockout and wild strains for changes in GSL production using LC-MS/MS analysis to identify the products of these enzymes.

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(P044) glycoRNA biology on the cell surface

Ryan Flynn[1]

[1]*Boston Children's Hospital,*

While RNA is not traditionally thought to be a major target of glycosylation, we have recently provided evidence that challenges this view. Using chemical and biochemical approaches, we found that conserved small noncoding RNAs bear sialylated glycans. These "glycoRNAs" were present in multiple cell types and mammalian species, in cultured cells, and in vivo. GlycoRNA assembly depends on canonical N-glycan biosynthetic machinery and results in structures enriched in sialic acid and fucose. Analysis of living cells revealed that the majority of glycoRNAs were present on the cell surface and can interact with anti-dsRNA antibodies and members of the Siglec receptor family. New chemical tools and insights into the molecular nature of glycoRNAs will be presented, which help to solidify the existence of a direct interface between RNA biology and glycobiology, and an expanded role for RNA in extracellular biology.

(P045) Identification of novel C-mannosylated proteins and its function

Siro Simizu[1]

[1]*Keio University, Japan,*

C-mannosylation is a unique protein glycosylation, and the roles remain largely unknown. We have successfully identified several *C*-mannosylated proteins and analyzed them in detail. As the results, we could suggest that *C*-mannosylation regulates protein stability, secretion, protein-protein interaction, intracellular trafficking and so on.

Isthmin-1 (ISM1) was identified as a secreted protein in *Xenopus* embryos. ISM1 contains a thrombospondin type 1 repeat (TSR1) domain in their central region. Phosphorylation of ISM1 has been reported, however whether or not human ISM1 is glycosylated has not been studied yet. Since ISM1 contains both two potential *N*-glycosylation and *C*-mannosylation sites, we examined whether ISM1 is glycosylated or not. We demonstrated that ISM1 is *C*-mannosylated at two Trp residues in TSR1 domain. These modifications affect intracellular localization and secretion of ISM1 and impedes the *N*-glycosylation of ISM1. Our results constitute the first report about a defect of *C*-mannosylation of a protein altering its *N*-glycosylation.

Premelanosome (PMEL) protein is a melanocyte-specific protein. In pigmented cells, PMEL forms amyloid fibrils in melanosomes, onto which melanin is deposited. Recent studies have reported that mutations of PMEL are associated with pigmentary glaucoma and oculocutaneous albinism in human, indicating PMEL in melanosome development for proper pigmentation. PMEL has one putative *C*-mannosylation site in its core amyloid fragment (CAF) region. We expressed recombinant PMEL in SK-MEL-28 human melanoma cells, purified and analyzed by MS. As a result, human PMEL is *C*-mannosylated at multiple tryptophan residues in its CAF and N-terminal fragment domain. To determine the effects of the modifications, we deleted the PMEL gene by using CRISPR/Cas9 technology and re-expressed wild-type or *C*-mannosylation-defective mutants of PMEL in SK-MEL-28 cells. Our results demonstrate that *C*-mannosylation of PMEL is required for proper melanosome development by regulating PMEL-derived fibril formation.

(P046) Paucimannose exposure mediated by lysosomal exocytosis

Morihisa Fujita[1], Wei-Wei Ren[1]

[1]*Institute for Glyco-core Research (iGCORE), Gifu University,*

Asparagine (N)-linked glycosylation is the most abundant post-translational modification of proteins. N-glycans are classified into three types based on their sugar composition and structures: high-mannose-type, hybrid-type, and complex-type. Among the high-mannose-type N-glycans, short N-glycans with three or fewer mannose residues are especially called "paucimannose". The paucimannose N-glycans are biosynthesized in protozoa, insects, and plants, whereas they are not formed in the normal biosynthetic process of mammalian cells. Recently, however, the presence of paucimannose has been observed in mammalian cells, and it is reported that paucimannose N-glycans are increased in several cancer cells. Mannitou, an antibody that recognizes paucimannose, weakly recognizes cell surface antigens, indicating that paucimannose can be expressed on the cell surface. However, limited information is available on the mechanism by which paucimannose-containing glycoproteins are formed and exposed on the cell surface. Since glycoproteomic analysis revealed that paucimannose is abundant on lysosome-localized proteins, we hypothesized that paucimannose is formed during N-glycan processing in lysosomes. In fact, the amounts of paucimannoses were decreased in cells lacking lysosomal glycoside hydrolases, HEXA and MAN2B1. Next, to clarify how the paucimannose N-glycans are exposed to the cell surface, we treated cells with various reagents. We found that the cell

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surface staining with Mannitou increased with inhibitors of mTOR, endocytosis, and calcium ionophores. On the other hand, Mannitou staining was decreased by Vacuolin-1, an inhibitor of lysosomal exocytosis, suggesting that paucimannose N-glycans are exposed to the cell surface via calcium-dependent lysosomal exocytosis. To identify key regulators of lysosomal exocytosis, we conducted a comprehensive genome-wide genetic screening to identify key regulators of lysosomal exocytosis. We revealed that an unconventional myosin plays a crucial role in controlling lysosomal exocytosis by stabilizing focal adhesions. Our results suggest the lysosomal exocytosis-mediated paucimannose exposure is regulated by focal adhesion stabilization.

(P047) Demonstration of biological significance of the unique C-domain in the vertebrate CMP-sialic acid synthetase

Di Wu[1], Sakura Toda[1,2], Masaya Hane[1,2,3], Chihiro SATO[1,2,3], Ken Kitajima[1,2,3]

[1]*Bioscience and Biotechnology Centre, Nagoya University,* [2]*Graduate School of Bioagricultural Sciences, Nagoya University,* [3]*Institute for Glyco-core Research, Nagoya University,*

CMP-Sialic acid synthetase (CSS) is prerequisite for the expression of Sia on the cell surface glycoconjugates, because it can only provide sialyltransferases with their substrate CMP-Sia. CSS thus activates free Sia to the corresponding CMP-Sia using CTP. The vertebrate CSSs consist of two domains, i.e., N- and C-terminal domains. The N-domain is a catalytic domain with the evolutionally conserved motifs, and also determines the preferential usage of the Sia species depending on animal species. In contrast, nothing has been known about the function of the C-domain except for its involvement in oligomerization of CSS. In this study, based on the following lines of evidence, we demonstrated the essential roles of C-domain for the first time. First, we generated a medaka strain (L304Q) with a single amino acid substitution in the C-domain, and found that most L304Q medaka were lethal within 19 days post-fertilization (dpf) [1]. The L304Q young fry displayed free Sia accumulation, and impairment of sialylation, up to 14 dpf. To demonstrate how this point mutation in C-domain of CSS impaired the enzymatic activity, L304Q and WT were characterized *in vitro*. As a result, L304Q and WT displayed a similar *in vitro* activity, while the thermostability of L304Q greatly decreased. These results suggest that L304 is important for the stability of CSS. Second, we also generated a mutant medaka with the C-domain-deleted CSS (ΔC-CSS) gene by the CRISPR-Cas9 system. Although the ΔC-CSS was even more active than wild-type CSS *in vitro*, ΔC-CSS medaka showed premature lethality, indicating that C-domain of vertebrate CSS is essential for animal surviving. Third, the real-time monitoring of CMP-Kdn produced by the rainbow trout CSS (rtCSS) was performed by ^{31}P-NMR. We found that that the C-domain of rtCSS affected the enzyme activity for Kdn [2]. Actually, CMP-Kdn inhibited the CMP-Neu5Ac production in a C-domain-dependent manner. These results also proved that the product inhibition of rtCSS-FL happened when Kdn was used as a substrate. We thus conclude that C-domain is involved in the feedback regulation of the CSS activity.

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(P048) The pro-inflammatory cytokines IL-6 and IL-1β induce ST6Gal1 expression within pancreatic cancer cells

Austin Silva[1], Susan Bellis[1]

[1]*Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham,*

The sialyltransferase ST6Gal1 is a key contributor to multiple diseases and malignancies. Studies from our lab have shown ST6Gal1 to be highly expressed within Pancreatic Ductal Adenocarcinoma (PDAC) and chronic pancreatitis, a major predisposing factor for PDAC. Interestingly, within pancreatitis tissues, ST6Gal1 is upregulated in cells undergoing acinar to ductal metaplasia (ADM), a process in which acinar cells de-differentiate towards a ductal, progenitor-like phenotype to heal the damage caused by pancreatitis. Furthermore, we reported that ST6Gal1 contributes to PDAC progression by imparting cancer stem cell characteristics. However, while ST6Gal1's phenotypic effects have been heavily investigated, the mechanisms underlying ST6GAL1 upregulation remain unclear. In the current study, we evaluated cytokine-induced expression of ST6GAL1 using the Suit-2 PDAC cell line and its metastatic subclones, S2-013 and S2-LM7AA. These cells show differential expression of ST6Gal1, with the metastatic subclones expressing ST6Gal1 at strikingly higher levels compared to Suit-2 cells. We treated these cell lines with a range of pro-inflammatory cytokines known to be released during PDAC and pancreatitis and determined that IL-6 and IL-1β stimulated stark upregulation of ST6Gal1 protein. Additionally, immunofluorescent staining of PDAC and pancreatitis tissues revealed that dysplastic cells with upregulated ST6GAL1 co-expressed IL-6 and IL-1β. We further assessed ST6Gal1 at the mRNA level and found that both IL-6 and IL-1β promoted significant increases in the "YZ" isoform of ST6Gal1, which is driven by the P3 promoter. This isoform is known to be ubiquitously expressed, while the "H" isoform and "X" isoform are selectively expressed by hepatocytes and B lymphocytes, respectively. In the PDAC cell lines, the YZ isoform was the major

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ST6GAL1 variant, although a very low level of the H isoform was also detected. We then examined the signaling pathways downstream of IL-6 and IL-1β. IL-6 activates the STAT3 pathway, while IL-1β activates NFκB. We therefore utilized inhibitors of STAT3 and NFκB and found that the inhibitors blocked cytokine-induced ST6Gal1 expression, implicating the IL-6/STAT3 and IL-1β/NFκB cascades in ST6Gal1 upregulation. Notably, the ST6Gal1 P3 promoter has multiple binding sites for STAT3 and NFκB. To determine if these transcription factors bind to the P3 promoter to drive transcription of the YZ isoform, we used a luciferase reporter construct containing the P3 promoter. This construct was transfected into cells, and cells were then treated with IL-6 or IL-1β. We observed that cells treated with IL-6 and IL-1β had significantly greater luminescence than untreated controls, confirming that these cytokines activated P3-dependent transcription. Taken together, these results highlight a novel molecular pathway by which cytokines within the tumor microenvironment stimulate the upregulation of ST6Gal1 in pancreatic cancer cells.

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(P049) 3'-Sialyllactose on Notch: Notch1 functions as a scaffold of O-linked, 3'-sialyllactosylated glycans

Yohei Tsukamoto[1], Kazuhiro Aoki[2], Yoshiki Narimatsu[3], Yusuke Urata[1], Wataru Saiki[1], Michael Tiemeyer[2], Henrik Clausen[3], Yuki Kurebayashi[5], Akira Minami[5], Tadanobu Takahashi[5], Tetsuya Okajima[1,4], Hideyuki Takeuchi[1,5]

[1]*Nagoya University School of Medicine,* [2]*University of Georgia/CCRC,* [3]*University of Copenhagen/CCG,* [4]*Nagoya University/iGCORE,* [5]*University of Shizuoka/School of Pharmaceutical Sciences,*

Notch signaling is important for the development and homeostasis of multicellular organisms. Genetic and biochemical studies have revealed that *O*-linked glycosylation on Notch receptors is essential for the transduction of Notch signaling. However, it is not fully understood how *O*-linked glycans regulate the activity of Notch signaling. To pursue this, we need to know the sites and structures of *O*-linked glycans on Notch receptors. Our mass spectrometric analysis of proteolytic digests derived from mouse Notch overexpressed in HEK293T cells revealed that many of the epidermal growth factor-like (EGF) repeats of Notch are modified with *O*-glucose (Glc), *O*-fucose (Fuc), and *O*-GlcNAc glycans at different stoichiometries. For example, protein *O*-glucosyltransferase 1 (POGLUT1)-dependent *O*-glucosylation occurs at a specific serine residue within a consensus sequence C^1-X-S-X-(P/A)-C^2 in folded EGF repeats. Surprisingly, we discovered sialylated, hexosylated *O*-Glc glycans specifically attached to the *O*-Glc site of EGF repeats within the ligand-binding region of Notch1 and 3. Further chemical analyses on β-elimination-released glycans indicated that the structure of the novel *O*-Glc glycans appeared to be Neu5Acα2-3Galβ1-4Glc-*O*. Genetic deletion of both *GXYLT1* and *GXYLT2* in HEK293T cells increased the ratio of the novel glycans on EGF repeats in Notch, suggesting that the xylosyl-extension suppresses the galactosyl-extension of *O*-Glc glycans at the specific EGF repeats. Knockout experiments targeting genes belonging to the B4 galactosyltransferase family or the ST3GAL family, either individually or in combination, identified a single galactosyltransferase B4GALT1 and a single sialyltransferase ST3GAL4 as the enzymes specifically responsible for the biosynthesis of this novel glycan. Furthermore, in many EGF repeats, an aromatic amino acid residue is conserved in the position two amino acids before the fourth cysteine residue, which is important for recognition by POGLUT1 and intramolecular interaction with the *O*-Glc glycan. Although this amino acid residue is apart from the *O*-Glc site in a primary sequence, it is closely located on the *O*-Glc site in properly folded EGF repeats. Notably, this aromatic amino acid is not conserved in EGF10 of Notch1, where we discovered a novel glycan chain. When we replaced the alanine residue at this position with an aromatic amino acid, the novel glycan disappeared. When we replaced it with alanine in EGF2 of NOTCH1, where the aromatic amino acid residue is conserved, the xylosyl-extension was reduced. Thus, both novel glycan biosynthesis and elongation of conventional type *O*-Glc glycans are dependent on the amino acid sequence of EGF repeats. This novel glycan may be present at the same location as the conventional *O*-Glc glycan and fine-tune Notch signaling. Supported in part by JSPS KAKENHI JP19H03176 and JP19KK0195 (to HT).

(P050) Role of O-GlcNAcylation in stress granule formation in cardiomyocytes undergoing chemical or ischemic challenges

Hannah Swimm[1], Wenxi Zhang[1], Yashas Mallikarjun[1], Deepthi Ashok[1], D. Brian Foster[1], Brian O'Rourke[1], Natasha E. Zachara[2], Kyriakos N. Papanicolaou[1]

[1]*Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD,* [2]*Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD,*

Background: The monosaccharide *O*-linked N-Acetylglucosamine (*O*-GlcNAc) modifies nucleocytoplasmic proteins on S/T residues. O-GlcNAcylation is regulated reversibly and dynamically by enzymes OGT (O-GlcNAc Transferase) and OGA (O-GlcNAcAse) which respectively add and remove O-GlcNAc. O-GlcNAcylation is a crucial regulator of cellular stress responses. Previous research found that O-GlcNAcylation is required for stress granule (SG) formation. SGs are cytoplasmic assemblies that form during acute stress by enclosing mRNA, translation initiation factors, ribosomal subunits, and proteins

important in protein-protein interactions and phase-separation. SG formation protects cells by rewiring translation, sequestering signaling proteins, maintaining the integrity of macromolecules, and preserving ATP levels. Evidence implicates SGs in neuronal dysfunction, but their role in cardiac muscle cells (cardiomyocytes) has remained unexplored. In the present work, we investigated if SGs form in cardiomyocytes under ischemic conditions and whether pharmacologic and genetic manipulation of O-GlcNAcylation alters SG formation.
Methods: G3BP1 is an RNA-binding protein that is essential for SG formation. We generated a novel adenovirus encoding the fusion protein G3BP1-EGFP to serve as a reporter for SG formation. This was expressed in cells of cardiac origin (H9c2) and monitored in real-time by confocal microscopy for 20 minutes after adding 500 μM sodium arsenite (SA). Data was analyzed offline using CellProfiler to determine the number of SGs per image. To pharmacologically manipulate O-GlcNAcylation, H9c2 cells were treated with the OGT inhibitor, OSMI-1, or the OGA inhibitors, Thiamet-G (TMG) and MK-8719. Further, siRNA transfection was performed to achieve the knockdown (KD) of OGT or OGA. Separately, primary cardiomyocytes (NRVMs) expressing EGFP-G3BP1 were similarly monitored during simulated ischemia using the application of a coverslip onto a monolayer of cells for 1 hour inducing acute hypoxia and nutrient deprivation.
Results: We observed rapid SG formation in H9c2 cells exposed to SA. Treatment with OSMI-1 significantly reduced SG formation. Similarly, OGT KD reduced SG formation. However, inhibition of OGA did not increase SG formation. This was corroborated in OGA KD cells. Next, we examined NRVMs after coverslip application and observed SG formation. Furthermore, these SGs exhibited strong O-GlcNAc immunoreactivity.
Conclusions: Baseline O-GlcNAcylation is necessary for SA-induced SG formation in cardiomyocytes, but its elevation does not increase SG formation. Critically, we found that simulated ischemia drives the formation of SGs containing O-GlcNAcylated proteins. Collectively, our data suggest that O-GlcNAcylation plays a crucial role in the formation of SGs in cardiomyocytes. Future studies should investigate if leveraging O-GlcNAcylation as a regulatory modality coordinating SGs can protect against cardiac ischemic injury.

(P051) Targeting Sialic Acids with Streptococcal Adhesin Proteins

KeAndreya Morrison[1,2], Barbara Bensing[3,4], Hai Yu[5], Xi Chen[5], Paul Sullam[3,4], Tina M. Iverson[2]

[1]School of Graduate Studies, [2]Department of Pharmacology, Vanderbilt University, Nashville, TN, [3]Division of Infectious Diseases, Veterans Affairs Medical Center, Department of Medicine, University of California, San Francisco, CA, USA, [4]the Northern California Institute for Research and Education, San Francisco, CA, USA, [5]Department of Chemistry, University of California, Davis, CA, USA,

Glycans terminating with sialic acid (sialoglycans) are important for many biological functions and are ubiquitous in both bacterial and mammalian cells. When found on mammalian cell surfaces one function is to act as ligands for sialic-acid-binding immunoglobulin-like lectins which suppresses autoimmunity. Likely linked to the attenuation of the immune response, aberrant glycosylation has been linked to initial oncogenic transformation and promotes tumor cell metastasis. This could make sialoglycan detection on cell surfaces a way to distinguish cancer cells. However, few techniques make glycan detection straightforward and high throughput. In ongoing work, we applied biochemical techniques to bacterial sialoglycan-binding proteins to build a library of probes specific for cancer-associated sialoglycans. The first target sialoglycans are 6-sulfo sialyl Lewis^X (6S-sLe^X) and sialyl Lewis^X (sLe^X), which are over-represented in colon and breast cancers. The starting scaffolds are Siglec-like binding region (SLBR) from *Streptococcus sanguinis* strain SK678 (SLBR$_{SK678}$) and *Streptococcus gordonii* strain UB10712 (SLBR$_{UB10712}$). SLBR$_{SK678}$ and SLBR$_{UB10712}$ are broadly selective and bind several Neu5Acα2-3gal containing glycans. The first round of engineering started with solving high-resolution crystal structures of parent SLBRs, *Streptococcus gordonii* strain Challis (Hsa) and *Streptococcus gordonii* strain M99 (GspB) bound with several sialoglycans. After structure evaluations and sequence comparison analysis with SLBR$_{UB10712}$ and SLBR$_{SK678}$, we created SLBR$_{UB10712}^{E285R/Q354D}$ and SLBR$_{SK678}^{E298R/Q367D}$, both of whom were predicted to be more selective for a single sialoglycan, 6S-sLe^X. Change in selectivity was tested with an ELISA assay where an almost 2-fold decrease in binding to other sialoglycans was measured. This indicates that SLBR$_{UB10712}^{E285R/Q354D}$ and SLBR$_{SK678}^{E298R/Q367D}$ are more selective for 6S-sLe^X than their wild-type forms. We also solved a 2.1-angstrom resolution structure of SLBR$_{UB10712}^{E285R/Q354D}$ using X-ray crystallography. In the second round, we continued with SLBR$_{SK678}$ due to its greater mutability. We developed new SLBR probes with sLe^X as our target. Using the same mutation creation method, we created five new mutations for SLBR$_{SK678}$, V297T, V297Y, V297H, V297D, and T309H. The valine and threonine residues were chosen because they interact closely and form hydrogen bonds with sialoglycans. They were swapped out for residues that would help facilitate binding to sLe^X while closing the binding site off to larger sialoglycans such as 6S-sLe^X. These are currently being tested with an ELISA assay against wildtype SLBR and the first-round mutants. We predict that these probes will be better binders for sLe^X, adding to our collection of probes. These findings are the starting point that will lead us toward a library of detection probes that could be used as a new screening technique for better detection and identification of sialoglycans.

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(P052) Galectin-8 protects renal function and decreases cell death in a model of acute kidney injury induced by folic acid

Elisa Perez-Moreno[1], Tomás Toledo[1], Teo Feuerhake[3], Gonzalo P. Méndez[4], Adely de la Peña[1,2], Francisca Pérez-Molina[1], Lorena Azócar[1], Alejandro Godoy[1,5], Pamela Ehrensfeld[6], Alfonso González[1,2], Andrea Soza[1,2]

[1]Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, [2]Centro Científico y Tecnológico de Excelencia Ciencia & Vida, [3]Department of Pathology, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile, [4]Pathology Unit, Laboratorio Inmunocel, Santiago, Chile, [5]Department of Urology, Roswell Park Comprehensive Cancer Center, Buffalo NY, 14263, [6]Instituto de Anatomía, Histología y Patología, Facultad de Medicina, Universidad Austral de Chile,

Acute kidney injury (AKI) is a public health problem characterized by an abrupt loss of kidney function, tubular cell death and inflammatory cell infiltration. Many pathological conditions can cause AKI, including renal hypoperfusion, sepsis, cardiorenal syndrome, and nephrotoxic exposure.

After an AKI, surviving proximal tubular cells dedifferentiate and proliferate to restore the tubular structures. However, the repair is often incomplete, leading to maladaptive repair that promotes the progression toward chronic kidney disease (CKD). Currently, there are no treatments that improve the outcome of those diseases. Therefore, understanding the molecular and cellular mechanisms of kidney repair can reveal new clues to improve the therapeutic intervention after AKI.

Galectin-8 (Gal-8) is a carbohydrate-binding protein that is secreted through an unconventional mechanism and binds to glycosylated proteins at the cell surface, promoting the activation of several signaling pathways. We previously described that Gal-8, which has immunosuppressive and neuroprotective potential, promotes epithelial cell dedifferentiation, proliferation and migration in Madin-Darby canine kidney (MDCK) cells. Here we evaluate the role of Gal-8 in kidney repair in a nephrotoxic mouse model of AKI induced by folic acid. Our results show that the intraperitoneal injection of Gal-8 24 hours before AKI induction protects renal function, measured by BUN and creatinine levels in plasma. Gal-8 also decreases the expression of the tubular damage markers KIM-1 and NGAL, and ameliorates the cortical damaged area as revealed by histological stains in kidney sections. Moreover, treatment with Gal-8 protects tubular epithelial cells from death as measured by TUNEL assay. Gal-8 significantly reduces the expression and the interstitial detection of fibrosis and myofibroblasts markers. Similar effects are observed when Gal-8 is injected 24 hours after AKI induction, supporting a possible role in acute events of kidney damage.

These results show that Gal-8 protects kidneys from nephrotoxic AKI, improving renal function and preventing epithelial tubular cell death, tissue damage and fibrosis.

Supported by FONDECYT#1211829 (AS), FONDECYT#1201635 (PE), FONDECYT # 1221067 (AG), FONDECYT # 1221796 (AG) AND Centro Ciencia & Vida, Basal FB 210008.

(P053) Galectin-8 Modulates Mitochondrial Dynamics in Epithelial Cells

Adely De la Pena[1], Claudio Retamal[1], Felix Randow[2], Alfonso Gonzalez[1,3], Claudia Oyanadel[1], Andrea Soza[1,3]

[1]Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencias, Universidad San Sebastián, [2]MRC Laboratory of Molecular Biology, [3]Centro Científico y Tecnológico de Excelencia Ciencia & Vida,

Galectin-8 belongs to a family of proteins that regulate a variety of cellular processes by interacting with beta-galactosidase moieties exposed in glycoproteins and glycolipids on the cell surface and in damaged endolysosomes. Galectin-8 has two carbohydrate recognition domains linked by a peptide chain and is unique among other galectins with a high preference for alpha-2,3 sialic acids within its N-terminal carbohydrate recognition domain (N-CRD). We previously demonstrated that treatment with recombinant Galectin-8 activates the Integrin-Focal adhesion Kinase (FAK)- epidermal growth factor receptor (EGFR) pathway, leading to ERK activation, proliferation, and migration in Madin-Darby Canine Kidney (MDCK) epithelial cells. Extensive research has established that ERK activation via the RAS-MAPK axis modulates mitochondrial dynamics, including fission, fusion, and distribution. Activation of ERK within the RAS-MAPK axis directly promotes mitochondrial fragmentation by activating DRP-1 and suppressing MFN1, leading to mitochondrial redistribution, reduction in calcium buffering, and activation of transcription factors that participate in tissue reparation. Identifying molecules that can modify mitochondrial dynamics is of great interest because they could enhance wound healing and tissue regeneration.Our study investigates how Galectin-8 affects mitochondrial dynamics via its carbohydrate-binding domain through the RAS-MAPK axis. Our results show that recombinant Galectin-8 induces fragmentation and perinuclear distribution of mitochondria in epithelial cells, promotes autophagy as indicated by the accumulation of LC3II over LC3I and decreased NDP52 levels, ultimately resulting in mitophagy. These effects are dependent on the EGFR/ERK pathway and the ability of Galectin-8 to bind cell surface carbohydrates through its N-terminal domain.

FONDECYT #1211829, 1221796, 21221618 and Centro Ciencia & Vida, Basal FB 210008.

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(P054) Regulation of mRNA Nuclear Export Efficiency by O-GlcNAcylation of Nuclear Pores

Samuel L. Junod[1], Weidong Yang[1]

[1]*Department of Biology, Temple University, Philadelphia, Pennsylvania, USA,*

The O-Linked beta-N-acetylglucosamine (O-GlcNAc) post-translational modification is prevalent in many nucleoporins (Nups) within mammalian cell nuclear pore complexes (NPCs). A significant proportion of these Nups are intrinsically disordered and rich in phenylalanine-glycine (FG) repeats. The O-GlcNAc modification predominantly occurs at the FG regions of these Nups and plays a crucial role in mediating the selective trafficking of macromolecules between the cytoplasm and the nucleus. Our study is focused on elucidating the impact of O-GlcNAcylated nuclear pore proteins on the export of mRNA from the nucleus through the NPCs. Leveraging high-speed super-resolution microscopy, we have discovered that the nuclear export efficiency of mRNAs can be either doubled or reduced to one third by modulating NPC O-GlcNAcylation levels in live cells. Furthermore, our spatial distribution three-dimensional mapping of FG Nups and their associated O-GlcNAc sites suggests that the regulation of mRNA nuclear export occurs through the modification of interactions between mRNA:protein complexes and the O-GlcNAc-modified FG Nups.

(P055) Development of 9-Pr4ManNAlk phenanthrene Derivative as Fluorescent-Raman Bioorthogonal Probe to Specifically interrogate the Biosynthesis of Gangliosides

Mana Mukherjee[1], Mathew Watson[2], Devin Biesbrock[1], Lara K. Abramowitz[1], Jennifer Lee[2], John A. Hanover[1,*]

[1]*LCMB, NIDDK, NIH, Bethesda, MD, USA,* [2]*NHLBI, NIH, Bethesda, MD, USA,*

Gangliosides are a group of sialic acid-containing glycosphingolipids that are an integral component of the cell membrane. Gangliosides are particularly abundant in the nervous system and involved in pathological conditions including cancer, neurodegenerative diseases, and storage disorders. The dedicated precursor for sialic acid biosynthesis is ManNAc, which can be epimerized from the corresponding UDP-GlcNAc or exogenously supplied ManNAc derivatives. The generated sialic acids are transferred to sphingolipids forming gangliosides. Currently, tools to visualize and detect gangliosides are very limited and none of them are specific. Bioorthogonal chemistry has been successfully implemented to detect sugars in vivo, and thus is an ideal tool to use for detection of gangliosides. Herein, we describe a dual fluorescent Raman active ManNAlk derivative, 9-Pr4ManNAlk phenanthrene (MM-JH-2), for the one-step specific labeling of gangliosides in cells. This modified ManNAlk derivative gives a unique Raman feature which allows unambiguous assignment within cells. Raman mapping of the probe co-maps with lipids. Using the fluorescent nature of this compound, we have been able to visualize and colocalize this probe with acidic compartments within cells. We used this bioorthogonal probe to differentiate between malignant and nonmalignant cells and found this probed promoted apoptosis in malignant cells. Further studies established that through specific incorporation and labelling of gangliosides, this compound can be used to distinguish B cells and T cells harvested from mice splenocyte.

(P056) Elucidating the Role of Adipocyte Heparan Sulfate in Fibroblast Growth Factor 1 (FGF1) Metabolic Signaling

Chung-Jui Yu[1], Marissa Maciej-Hulme[1], Nathan Thomas[1], Shihao Liu[2], Michael Downes[2], Ronald Evans[2], Philip Gordts[1,3]

[1]*Division of Endocrinology and Metabolism, School of Medicine, University of California, San Diego,* [2]*Salk Institute, Gene Expression Laboratory, La Jolla, CA,* [3]*Glycobiology Research and Training Center, University of California, San Diego,*

Type 2 diabetes mellitus (T2DM) is a major public health issue that affects nearly 9% of the adult population worldwide, resulting in yearly expenditures nearly $825 billion USD on T2DM management alone. Pathogenesis of T2DM involves the development of insulin resistance and pancreatic β-cell impairment that engenders hyperglycemia, a condition that further amplifies the aforementioned abnormalities and metabolic glucose metabolism. Therapeutic approaches that induce and restore sustained glycemic control can serve to inhibit T2DM pathogenesis and mitigate complications arising from hyperglycemia. Fibroblast growth factor 1 (FGF1) has demonstrated enormous promise as a key metabolic hormone in the management of nutrient stress and glycemic control. Recently, FGF1 has shown to suppress lipolytic activity in murine adipose tissue by inducing phosphorylation of phosphodiestertases that inhibits cyclic AMP (cAMP)-dependent lipolysis, thereby suppressing hepatic gluconeogenesis. To further understand the anti-hyperglycemic action of FGF1 in adipocytes, we sought to investigate the role of heparan sulfate (HS) proteoglycans in the FGF1 signaling pathway. *In vitro* experiments on mouse embryonic fibroblasts

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(MEFs)-derived adipocytes with and without heparinase treatments demonstrate surface HS as crucial mediators for FGF1 signaling to trigger glucose uptake and anti-lipolytic activity. We investigate the natural variability in HS sulfation in human adipose tissue and the associated FGF1 binding affinities, which have implications in the broad efficacy of the FGF1 pathway as an anti-diabetic therapy. The sum of this work establishes the fundamental role of HS and its natural variation in FGF1-induced anti-lipolytic and glycemic control in adipocytes.

(P057) Unraveling the Sugary Side of Notch: How overexpressed Fringe proteins shape osteoclastogenesis

Sylvia Kennerly[1], Jason Ashley[1]

[1]*Eastern Washington University,*

Osteoclasts, the multinucleated cells crucial for bone resorption, are a critical component in both physiological and pathological skeletal remodeling. Osteoclastogenesis, the process of differentiation of the monocyte/macrophage originating precursors to osteoclasts, relies heavily on RANK signaling. However, the size, activity, and lifespan can be influenced by other pathways, such as Notch signaling. Notably, Notch signaling is regulated by selective glycosylation through the Fringe family of O-fucose-specific ß1,3-N-acetylglucosaminyltransferases (O ß1,3 GnTs). To investigate the impact of individual Fringe glycosyltransferases on osteoclastogenesis, we conducted experiments exploring the overexpression of the individual Fringe glycosyltransferases [Lunatic (LFNG), Manic (MFNG), and Radical (RFNG)] found to influence mammal Notch pathways. These full-length coding sequences were obtained from a mouse bone marrow cDNA library and inserted into the pMXs-Puro vector, the Moloney Murine Leukemia Virus (MMLV)-derived gammaretroviral system. Retroviral particles were generated through transient co-transfection with VSV-G envelope plasmids into Platinum-E packaging cells. Primary bone marrow cells collected from male C57BL/6 mice were infected with Fringe plasmids and subjected to variable seeding methods with RANKL and MCSF exposure to stimulate osteoclastogenesis. At the conclusions of differentiation, we stained cells with acridine orange (AO) stain as well as for tartrate-resistant Acid Phosphatase (TRAP) activity to assess resulting morphological variations. Preliminary results found that LFNG-overexpressing (OE) and MFNG-OE cell lines in primary cultures showed increased osteoclast formation while RFNG-OE lines showed decreased osteoclastogenesis. These initial results suggest the need for further experimentation to understand the downstream impact on cell lifespan, physiology, and function.

(P058) Combination of CRISPR screens and lectin microarrays enables identification of novel cell surface glycosylation regulators

C. Kimberly Tsui[1], Nicholas Twells[2], Emma Doan[1], Janaiya Brooks[1], Ayodeji Kulepa[2], Brant Webster[1], Lara K. Mahal[2], Andrew Dillin[1]

[1]*University of California, Berkeley, Department of Molecular Cell Biology, Howard Hughes Medical Institute,* [2]*University of Alberta, Department of Chemistry,*

Glycans on the cell surface regulate cellular signaling and function and are part of the language by which cells communicate. Unlike proteins, glycan structures are not templated from genetic sequences but the concerted activity of many genes. While the biosynthetic enzymes directly responsible for producing specific glycan structures are largely characterized, little is known about the expanded network of genes that control the expression and localization of those enzymes, glycan trafficking, organelle function, substrate availability, and cellular response pathways that regulate the repertoire of glycans that reach the cell surface. Here, we present a strategy that pairs pooled CRISPR screens with lectin microarrays, allowing us to identify and characterize novel genetic regulators of cell surface glycosylation beyond the known biosynthetic enzymes.To illustrate this, we applied our approach to uncover regulators of high mannose N-glycans, which are the base structure of all N-glycans and can act as a stress signal during viral infection. We first used CRISPR screens to identify the network of genes controlling the presence of this glycan on the cell surface, followed by lectin microarrays to fully measure the complex effect of selected regulators on glycosylation globally. Through this, we discovered the role of CCDC22/Commander complex as regulators of the trans-Golgi network and of N-glycan elongation and α2,3-sialylation, and elucidated the role of previously uncharacterized gene TM9SF3 as a regulator of cis-Golgi morphology and complex N-glycans formation. This work identifies regulators of high mannose N-glycosylation and represents a generalizable approach for systematically dissecting the regulatory network that underlies cell surface glycosylation.

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(P059) ST6GAL1 insufficiency accelerates hematopoietic stem cell loss following acute bone marrow radiation syndrome

Ogechi Ogoke[1], Amelia Colder[1], Valerie Andersen[1], Amy Freeland[1], Michael J. Nemeth[3], Karin Hoffmeister[2], Joseph T.Y. Lau[1]

[1]*Department of Molecular and Cellular Biology, Roswell Park Comprehensive Cancer Center, Buffalo New York,* [2]*Translational Glycomics Center, Versiti Blood Research Institute, Milwaukee, Wisconsin USA,* [3]*Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo New York,*

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Acute radiation syndrome (ARS) originates via exposure to harmful level of radioactive stimuli driving organ tissue failure and eventual mortality. Organ systems most sensitive to irreversible radiation injury include: (1) the gastrointestinal system, where mortality ensues days after lethal dose irradiation and (2) the hematopoietic system, whereby mortality eventually develops from the inability to replenish multilineage blood cells populations. Previously we have showed that the inability to express functional ST6GAL1, which mediates α2,6-sialylation of Gal (β1,4) GlcNAc glycan termini, renders increased sensitivity to ionizing radiation in mouse models. Mortality of *St6gal1*-null animals, by radiation resulted from irreparable damage to the gastrointestinal tract, putatively to the intestinal stem cells necessary for the continuous regeneration of the gut epithelium. While clear that hematopoiesis is also damaged by radiation, it is not known whether functional ST6GAL1 protects the marrow from radiation injury. Here, we evaluate how ST6GAL1 contributes to the earliest events in hematopoiesis following irradiation *in vivo*. Mice compared between age/sex matched controls, were exposed to a reduced radiation regiment (3 Gy) and observed for 5 days post irradiation. At baseline, similar cell numbers of hematopoietic progenitors defined as lineage^{neg}; c-KIT^{pos}; Sca-1^{pos} (LSK) were present in *St6gal1*-null and control *wild-type* C57BL/6 marrow. However, 5 days after exposure to 3Gy irradiation, we observed a 2-fold reduction in LSK numbers in the *St6gal1*-null compared to the wild-type marrow. LSK cells from wild-type marrow had strikingly increased proportion of SNA^{pos} cells, strongly suggesting involvement of ST6GAL1 during re-establishment of hematopoietic homeostasis. The LSK pool was further dissected to examine the contributing subpopulations including long-term and short-term hematopoietic stem cells (LT-HSC, ST-HSC), and multipotent progenitors (MPP). We observed significant decreases in cell numbers of MPP and ST-HSCs of the *St6gal1*-null mouse. Interestingly, LT-HSCs did not demonstrate significant changes in cell count between wild-type and the *St6gal1*-null mouse suggesting non-reliance on native ST6GAL1 expression for that specific marrow compartment. To examine the effect on HSCs directly, bone marrows were flushed from *St6gal1*-null and wild-type mice at baseline. Hematopoietic progenitors were enriched from marrow extracts prior to irradiation at 0.5 Gy and culture *in vitro*. Cell proliferation 5 days after irradiation *ex vivo* was monitored, where we observed a significantly reduced amount of progenitor cells in *St6gal1*-null cultures compared to wild-type. Together, our data point to a role for functional *St6gal1* in damage restoration of not only the gastrointestinal system, but the hematopoiesis system, after radiation injury.

(P060) A hidden player: the influence of transmembrane domain of α-Glucosidase I in a fission yeast MOGS-CDG model

Tommy Idrovo-Hidalgo[1], Sofia Orioli[1], Cecilia D'Alessio[1,2]

[1]*Instituto de Biociencias, Biotecnología y Biología Traslacional (iB3), Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,* [2]*CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas),*

N-glycosylation is a highly conserved process among eukaryotes and represents one of the most relevant posttranslational modifications in the secretory pathway. It starts with the transfer of a pre-assembled oligosaccharide $Glc_3Man_9GlcNAc_2$ (G3M9) in the endoplasmic reticulum (ER) membrane to asparagine residues of a consensus sequence of proteins entering the ER. Immediately, the ER- glucosidase I (GI) trims the outermost glucose of glycoproteins converting G3M9 into G2M9 and glucosidase II (GII) generates G1M9-bearing glycoproteins. These are recognized by calnexin/calreticulin lectins involved in the endoplasmic reticulum quality control of glycoprotein folding mechanism (ERQC), which ensures that only proper folded glycoproteins continue the secretory pathway. GI associated defects produce congenital disorders of glycosylation (CDG) type IIb (or MOGS-CDG). This disease manifests with multisystemic failures at different ages in human patients. It has been stated that unactive GI fails to trim G3M9 into G2M9 and our previous findings demonstrated that the accumulation of G3M9 glycoproteins due to the lack of GI in *Schizosaccharomyces pombe* (ΔGI mutants), is extremely toxic for the cell. ΔGI yeast mutants are shortened and clumped, with a slow cell growth and a low viability. This sick phenotype could be partially -but not fully- suppressed by knocking out an alpha-1,2-glucosyltransferase (alg10p) that adds the outermost residue of glucose during glycan biosynthesis, the same glucose that is a substrate for GI. On the other hand, most mutations found in patients are not within the proposed catalytic pair residues of GI. Both results could imply that not only the catalytic function of GI is involved in the sick phenotype of cells lacking GI, but that other domain/s could also be important. Interestingly, while GII is a soluble protein of the ER lumen, GI is an ER membrane bound protein that contains three domains: a globular catalytic domain inside the ER lumen, a transmembrane domain, and a short cytosolic tail. In this study, we examined the impact of expressing a soluble catalytic-domain-only GI (cGI) in fission yeast ΔGI ER lumen. We verified the proper expression and localization of

the cGI variant by western blot and confocal fluorescence microscopy. We also confirmed the presence of GI activity by an *in vitro* fluorometric assay that detects glucose hydrolysis from a GI-specific synthetic substrate. Even though the cells express GI activity in the ER lumen the cell viability, growth rates and cell lengths are similar to those in ΔGI cells. Our results indicate that the expression of an active cGI in the ER lumen is not able to rescue the sick phenotype of cells lacking the full gene coding GI, supporting the hypothesis that GI may have an undiscovered additional role, possibly related to its ER transmembrane domain.

(P061) Colony Stimulating Factor 1 Receptor Sialylation as a Possible Target for Modulation of Osteoclastogenesis

Eric Beaulaurier[1], Jason Ashley[1]

[1]*Department of Biology, Eastern Washington University, Cheney, Washington, USA,*

Colony stimulating factor 1 receptor (CSF1R) is ubiquitous across monocytes, macrophages, and osteoclasts and provides survival and proliferation signals. CSF1R is also vital to priming osteoclast precursors for differentiation in both the canonical (RANKL dependent) and non-canonical pathways (RANKL independent). Interestingly, CSF1R has two ligands, colony stimulating factor 1 (CSF1) and interleukin 34 (IL-34), which produce similar but distinct downstream effects due to the flexibility of the receptor as well as variable affinities in the binding of CSF1:CS1R and IL-34:CSF1R complexes. Due to distinct sources of the ligands and variation in downstream effects, disease states associated with each ligand are distinct, though many share an osteolytic component. It has previously been observed that CSF1R bears sialic acid moieties on its extracellular region; however, neither the specific orientation (α2,3 or α2,6), nor function of CSF1R sialylation have been elucidated. Though this receptor had long been of interest in the treatment of osteolytic disease, the severe osteopetrotic, immunosuppressive, and developmental implications associated with lack of CSF1R signaling has kept It from being a viable therapeutic target. It is the aim of our study to determine if manipulation of CSF1R's terminal sialic acids would allow for modulation of signaling sensitivity between its two ligands. Investigation of CSF1R sialylation proves difficult due to the limited specificity of commercially available neuraminidase, but through comparative use of multiple exogenous neuraminidases and lectin probing, we have determined that CSF1R sialylation is present in a α2,6 orientation. Additionally, our preliminary work suggests that CSF1R sialylation significantly impacts osteoclast precursor survival and differentiation.

(P062) Effect of uridine diphosphate on the enzymatic activities of UDP-GlcA decarboxylase, xylosyltransferase, and glucuronyltransferase

Shuji Mizumoto[1], Asaka Takayasu[1], Kenji Furukawa[1], Shuhei Yamada[1]

[1]*Meijo University, Japan,*

Glycosaminoglycans (GAGs) are ubiquitously present on cell surfaces and in extracellular matrices as sugar side chains of proteoglycans, and involved in various biological events such as cell differentiation and proliferation by interacting with bioactive proteins. GAG side chains are biosynthesized on serine residues in the core proteins through the common GAG-protein linkage structure, glucuronic acid (GlcA)-galactose-galactose-xylose (Xyl). The first and forth steps in the biosynthesis of this tetrasaccharide linkage region are catalyzed by xylosyltransferase (XylT) and glucuronyltransferase-I (GlcAT-I), which transfer Xyl and GlcA residues from uridine diphosphate (UDP)-Xyl and -GlcA, respectively. UDP-GlcA incorporates into endoplasmic reticulum (ER) or Golgi apparatus from cytosol. On the other hand, UDP-Xyl is synthesized from UDP-GlcA by UDP-GlcA decarboxylase (UGD). After the enzymatic reactions by glycosyltransferases, the reaction product, UDP, is converted into uridine monophosphate (UMP) by calcium-dependent nucleotidase 1 (CANT1). Thereafter, UMP is exported to cytosol through an antiporter, nucleotide sugar transporter. Mutations in xylosyltransferase 1 (XYLT1), glucuronyltransferase-I (GlcAT-I), or CANT1 cause Desbuquois dysplasia type 2 or pseudodiastrophic dysplasia [1–3]. The mutation of CANT1 results in defect in conversion from UDP to UMP, leading to an accumulation of UDP in the ER or Golgi apparatus, which may disturb XYLT1, GlcAT-I, or UGD. In the present study, inhibition of XYLT1, GlcAT-I, and UGD enzymatic activities by UDP was examined. Expression vectors containing the genes encoding XYLT1, GlcAT-I, and UGD proteins were constructed and transfected into COS-7 cells to transiently express the recombinant proteins. The conditioned medium was collected, and the enzymatic reaction was performed using the purified protein as the enzyme source, fluorescently labeled decorin-peptide, 4-methylumbelliferyl-galactose, or UDP-GlcA as the substrate. The reaction products were purified by gel filtration or anion-exchange chromatography. These activities of XYLT1, GlcAT-I, and UGD were significantly decreased in the presence of UDP as an inhibitor. Therefore, in patients with CANT1 mutations, the accumulated UDP in the ER or Golgi apparatus may inhibit the glycosyltransferase reactions of XYLT1 and GlcAT-I or UGD reaction, which leads to partial defects in GAGs, resulting in the common hereditary skeletal disorders.

[1] Bui et al., Am J Hum Genet, 94, 405, 2014.
[2] Byrne, Mizumoto et al., J Med Genet, 57, 454, 2020.
[3] Huber et al., Am J Hum Genet, 85, 706, 2009.

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(P063) Identification and characterization of a novel deaminoneuraminic acid (Kdn)-specific aldolase from Sphingobacterium species

Takahiro Nakagawa[1], Yuya Iwaki[1,2], Di Wu[1,2,3], Masaya Hane[1,2,3], Chihiro Sato[1,2,3], Ken Kitajima[1,2,3]

[1]*Biosci. Biotech. Center, Nagoya Univ.*, [2]*Grad. Sch. Bioagr. Sci., Nagoya Univ.*, [3]*iGCORE, Nagoya Univ.*,

Sialic acids (Sias) are a group of acidic sugars with a nine-carbon backbone and classified into three molecular species, based on substituent groups at the C5 position; *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (Kdn). Sias are located at the termini of glycans and involved in various biological phenomena such as fertilization, development, tumorigenesis and immune response. In bacteria, Sias are often used as nutrients through incorporation and subsequent catabolic reactions. First, the terminal Neu5Ac residues are hydrolyzed by sialidase, and then the liberated Neu5Ac is cleaved into *N*-acetylmannosamine (ManNAc) and pyruvate by sialate-pyruvate lyase or *N*-acetylneuraminate aldolase (NanA). In contrast with Neu5Ac-glycans, most bacteria cannot utilize Kdn-glycans because Kdn-glycans are resistant to sialidases. However, we previously found a Kdn-specific sialidase that can hydrolyze the Kdn-glycosides, but not Neu5Ac-glycosides in a Kdn-glycans assimilating *Sphingobacterium* sp. isolated from soils. We thus wondered if the sialate pyruvate lyase from this bacterium was also Kdn-specific, and this study sought to characterize the enzymes. Whole-genome sequencing of this bacterium revealed that there were four aldolase candidates; ORF1728, ORF2211, ORF4424 and ORF4464. They were then expressed using *Escherichia coli*, and the enzymatic properties were analyzed. The results showed that the ORF2211 was Kdn-specific aldolase (Kdn-aldolase). Kdn-aldolase had the optimal pH and temperature at 7.0–8.0 and 50 °C, respectively. In addition, Kdn-aldolase had Kdn synthetic activity. The molecular modeling and site-specific mutagenesis revealed that the Asn50 in Kdn-aldolase was important for Kdn-recognition. These results indicated that both of the sialidase and the sialate pyruvate lyase were highly Kdn-specific, suggesting that, apart from Neu5Ac catabolism, the Kdn-specific catabolism is important in nature.

(P064) O-glycans modulate the interaction between CD45 and B cell receptor complex

Po-Long Hsiao[1], Ho-Yang Tsai[1,2,3], Shih-Yun Guu[1], Wan-Chen Huang[4], Kay-Hooi Khoo[1,2], Kuo-I Lin[3], Takashi Angata[1,2]

[1]*Institute of Biological Chemistry, Academia Sinica*, [2]*Institute of Biochemical Sciences, National Taiwan University*, [3]*Genomics Research Center, Academia Sinica*, [4]*Institute of Cellular and Organismic Biology, Academia Sinica*

B cells play an essential role in immunity by differentiating into plasma cells that produce antibodies. Whereas the importance of N-glycosylation in regulating B-cell differentiation and function has been well documented, the role of O-glycosylation in B-cell function has gained attention only recently. O-glycans undergo major structural changes during B-cell differentiation, implying such structural changes may be important for fine-tuning B-cell differentiation. To investigate whether O-glycans modulate the interaction between B cell receptor (BCR) and co-receptor(s) and ensuing BCR signaling, we developed a simplified system based on the mouse myeloma J558L cell line, which lacks some components of the BCR complex and several co-receptors including CD45, a tyrosine phosphatase that is heavily O-glycosylated and plays a crucial role in BCR signaling. J558L cells were reconstituted with the missing BCR components and CD45 fused with orthogonal self-labeling protein tags, and enzymes that modify O-glycans were further introduced. The cells were subjected to Förster resonance energy transfer-based analysis of BCR–CD45 interaction, as well as the evaluation of BCR signaling by Western blotting. Our study revealed that the addition of α2–8-linked sialic acids by ST8Sia6 reduces the distance between BCR and CD45 and facilitates BCR signaling, whereas the branching of O-glycans by GCNT1 has a negligible effect on BCR–CD45 interaction and BCR signaling. Our study revealed differential modulation of the BCR–CD45 interaction by structurally distinct O-glycans, demonstrating the utility of the system for the functional evaluation of O-glycans in BCR signaling.

(P065) The human ganglioside interactome in live cells revealed using clickable photoaffinity ganglioside probes

Gao-Lan Zhang[1], Mitchell J. Porter[1], Abduselam K. Awol[1], Benjamin C. Orsburn[1], Robert N. O'Meally[2], Robert N. Cole[2], Ronald L. Schnaar[1]

[1]*Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA*, [2]*Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA*,

Gangliosides, sialic acid bearing glycosphingolipids, are functional glycans on the outer leaflet of the plasma membranes of all vertebrate cells. Altered ganglioside expression is associated with diseases including cancer, diabetes, microbial pathogenesis and severe cognitive deficits. Ganglioside regulate cell physiology by interacting with proteins on their own membranes (*cis*) or

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in the extracellular milieu (*trans*). As anionic amphiphiles prone to non-specific binding, affinity identification of ganglioside interacting proteins (the ganglioside interactome) has been challenging. To address this, we developed minimally disruptive ganglioside probes carrying a bifunctional tag with a photoreactive diazirine and a click-reactive alkyne. Bifunctional tags were chemically installed on the ganglioside ceramide, at the C7 of the sialic acid glycerol side chain, or at the C6 of the terminal galactose. Efficient methods were optimized to deliver tagged gangliosides (GM3, GM1, GD1a) to the outer leaflet of the plasma membrane of human epidermoid carcinoma cells (A-431) and human neuroblastoma cells (SH-SY5Y). Cells were then UV irradiated to crosslink the diazirine to ganglioside-interacting proteins in live cells followed by solubilization, click biotinylation and Neutravidin capture. Captured proteins were subjected to on-bead digestion and released peptides were analyzed by isobaric tandem mass tag relative quantitative proteomic analysis. Hundreds of proteins were highly enriched in probe-treated samples, with binding interactions dependent on the ganglioside used. Both previously implicated and novel ganglioside interactors were identified. Gene ontology revealed that >90% of the proteins captured using ceramide-tagged gangliosides mapped to the plasma membrane, whereas glycan-tagged gangliosides captured proteins in the extracellular milieu. Molecular Function analysis revealed that ganglioside-interacting proteins were highly significantly overrepresented by transmembrane transporters and different classes of cell adhesion molecules. These novel bifunctional probes facilitated the identification of the ganglioside interactome in multiple human cells and unveiled binding protein specificity among different gangliosides. Both *cis* and *trans* interacting proteins were captured. The reported methods are applicable to other gangliosides and cell types, promising to provide insights into the molecular and cellular regulation by gangliosides. Supp by NIH grant U01 CA241953.

(P066) Cationic coordination of Cryptococcus neoformans capsular polysaccharide results in cellular aggregation

Gracen Gerbig[1], Maggie Wear[1], Jason Sonnenberg, Arturo Casadevall[1]

[1]*Johns Hopkins Bloomberg School of Public Health,*

Cryptococcus neoformans is an environmental yeast with a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM) that facilitates survival in a variety of environments including human hosts and was recently top ranked by the World Health Organization as a critical priority pathogen given its importance as a cause of human disease. Of particular interest is how the polysaccharide capsule facilitates cellular interactions with abiotic and biotic stimuli. Previous studies have suggested that calcium forms divalent bridges in the capsule, but how this contributes to capsular architecture as well as calcium's contribution to the hydrophilic capsule overcoming cell-cell repulsion to aggregate have not been explored. Here, we present new data about how the additive effects of calcium on cryptococcal polysaccharides lead to cellular aggregation. We hypothesize that the coordination of calcium's orbitals favors between four and eight oxygen interactions, suggesting that GXM cationic bridging involves more than two carboxyl groups, resulting in multi-glycan coordination with the calcium metal. We characterized how divalent cations affected GXM by complimentary techniques using two serotype A strains of *C. neoformans*. We performed light microscopy to evaluate cellular aggregation/biofilm formation, Dynamic Light Scattering (DLS) analysis to characterize the size of polysaccharide aggregates, Attenuated Total Reflectance spectroscopy (ATR) to define the residues involved in ionic bridging, and electronic structure theory (EST) simulation to model the energetics of cation-polysaccharide interactions and define the molecular interaction within GXM. Screening of four cations—magnesium, calcium, strontium and cadmium—showed selective cellular aggregation within soluble concentrations exclusively with calcium (>0.5 M). This extends from heat killed cells to isolated capsular polysaccharide and is reversible with ethylenediaminetetraacetic acid (EDTA) chelation. Previous work by our lab showed that capsule assembly requires divalent cation coordination of GXM (optimal 0.03 mM $CaCl_2$). Together this data suggests that calcium interaction in the capsule of *C. neoformans* is more complex than previously theorized. Not only does divalent cation bridging occur within the capsule, but also between the capsules of individual cells, though these two phenomena occur at different calcium concentrations. These observations yield new insights into the assembly and architecture of the capsule, an essential virulence factor for *C. neoformans*. Additionally, from the perspective of human infection, aggregated cell clumps, which are common on tissue histology, are not readily phagocytosed, suggesting that calcium-dependent aggregation may present an advantage for survival in mammalian hosts.

(P067) Localization and identification of 3-O-sulfated galactosylated proteins in murine brain using recombinant lamprey antibody

Maxence Noel[1], Suttipong Suttapitugsakul[1], Lukas Muerner[1], Dongli Lu[1], Ea Tulin[1], Robert G. Mealer[2], Richard D. Cummings[1]

[1]*National Center for Functional Glycomics, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA,* [2]*Department of Psychiatry, Oregon Health & Science University, Portland, OR,*

Glycan sulfation is a post-translational modification occurring in various glycan structures but its precise role in the brain is still not fully understood. Our recent results focusing on mapping the brain N- and O-glycoproteomes and glycan expression using

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a panel of lectins suggested that 3-O-sulfated galactose (3-O-SGal) is highly present in the synapse-rich layer of the cerebellum. However, the lability and abundance of the modification makes it difficult to study by mass spectrometry and the lack of specificity of lectins for sulfate modifications of glycans highlight the need for more specific reagents.To confirm the presence of 3-O-SGal in the brain and increase the specificity, we used a monoclonal antibody O6 that was produced in immunized lampreys and is specific to this modification. This approach allowed us to precisely localize 3-O-SGal-containing structures within murine brain slices using immunofluorescence technique and perform enrichment approaches to identify their protein carriers through proteomics. In this study, we show that 1) the 3-O-SGal modification is highly abundant in the dendritic tree of the Purkinje cells, the Ammon's horn of the hippocampus, and a specific layer in the cortex; 2) 3-O-SGal occurs mostly on N-glycans; 3) the glycoproteins carriers are associated with cell-cell adhesion, pre- and post-synaptic compartment and ion channels in neurons; and 4) by using the Neuro-2a cell line that expresses 3-O-SGal, transcriptomic analyses, and a CRISPR/Cas9-deletion approach, we identified GAL3ST3 as the gene encoding the Gal3ST3 enzyme likely responsible for formation of the 3-O-SGal epitope.This study is one of the first to deeply characterize the 3-O-sulfation of galactose and demonstrates its unique expression pattern in the brain. The results suggest critical roles for this modification in glycoprotein functions. Future studies are focusing on further identifying the specific cells and functions of glycoproteins expressing this modification.

(P068) Evaluation of PIGN-CDG-related defects in GPI anchor synthesis and ER-related protein aggregation

Rashmi Yadav[1], Andrew Edmondson[1]

[1]*Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA, USA,*

Glycosylation involves the construction of carbohydrate structures from monosaccharides and their attachment to proteins as a post-translational modification. It plays critical roles in protein processing and quality control, subcellular localization, and protein function. Defects in glycosylation cause a group of genetic disorders known as Congenital Disorders of Glycosylation (CDG). To date, approximately 170 genetic causes of CDG have been discovered. PIGN-CDG is a disorder of glycosylphosphatidylinositol (GPI)-anchor biosynthesis. PIGN (Phosphatidylinositol Glycan Anchor Biosynthesis Class N) transfers ethanolamine phosphate to mannose in the process of GPI-anchor synthesis and has also been shown to play a role in preventing protein aggregation in the endoplasmic reticulum (ER). Disruption of GPI-anchor synthesis reduces cell surface localization of GPI-anchored proteins, which play important roles in cell adhesion, signal transduction, and antigen presentation. PIGN-CDG patients have neurologic dysfunction, including severe developmental delay, hypotonia, and seizures, and severely affected patients may have congenital anomalies, including congenital diaphragmatic hernia, congenital heart disease and renal anomalies. PIGN-CDG is typically diagnosed through genetic testing, but analysis of the cell surface localization of GPI-anchored proteins may help with resolving genetic variants of uncertain significance. Additionally, evaluating the impact of genetic variants on PIGN-related functions of GPI-anchored protein cell surface expression and ER protein aggregation may help to resolve genotype/phenotype correlations, providing diagnostic and prognostic information. We have evaluated patient-derived fibroblasts from a series of 7 PIGN-CDG patients with a range of clinical severity for cell surface expression of GPI-anchored proteins (Cd59, Cd16, FLAER, RECK, Thy1) using flow cytometry and for evidence of ER stress and upregulation of unfolded protein response genes (*HSPA5*, *ATF4*, *OS9* and *HSP90B1)* by qPCR. Our study furthers our knowledge of how PIGN-CDG-related pathogenic variants impair GPI-anchor synthesis and affect PIGN cellular functions.

(P069) Structural and functional profiling of the Glycoprotein NMB heparan binding site

Nathan Thomas[1], Chung-Jui Yu, Pranav Madabhushi[1], Marissa Maciej-Hulme[1], L.S.M. Gordts[1]

[1]*Glycobiology Research and Training Center, Department of Medicine, University of California, San Diego,*

Glycoprotein NMB (GPNMB) is a protein expressed by innate immune cells that has been implicated in numerous diseases hallmarked by chronic inflammation, including nonalcoholic steatohepatitis, neurodegeneration, tumor progression, and heart failure. Plasma levels of GPNMB are inversely correlated with myocardial infarction and are associated with adverse recovery outcomes through an unknown mechanism. Despite this medical significance, little is known about GPNMB's biological mechanism. Our work is focused on structurally and biochemically characterizing GPNMB and its putative heparan sulfate binding site through heparin binding chromatography, signaling assays, and structural simulations to better understand its role in health and disease. The ectodomain of GPNMB (ecGPNMB) consists of an N-terminal IgG-like domain (Domain 1), a central PKD domain that contains a proline-rich loop (Domain 2), and an N-terminal Kringle-like domain (Domain 3). To probe the function of these domains, we designed constructs in which each domain is deleted (including a construct missing only the loop) and in which each domain is expressed by itself. All constructs have been successfully cloned and most properly express. Our initial surface charge modeling using the AlphaFold predicted structure and molecular dynamics suggests that Domain 1 has a highly positive patch likely to be responsible for binding to heparan sulfate. Heparin binding *in vitro* has been confirmed and minimal oligo length defined using differential scanning fluorimetry. Work is now underway using *in silico* molecular docking

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and mutagenesis analysis to define the heparan binding sites and assess the activity of each of mutant construct in inducing GPCR-mediated signaling. The structural and mechanistic information obtained in this project will provide crucial information for understanding the role of GPNMB in heart disease.

(P070) Methods and applications of electron-based dissociation (ExD) methods for analysis of glycan roles in cell biology

Catherine E. Costello[1], Chaoshuang Xia[1], Juan Wei[1,2], Nadia Cherkassky[1], M. Maissa Gaye[1], Cheng Lin[1]

[1]*Boston University Chobanian & Avedisian School of Medicine, Boston, MA 02118-2646 USA,* [2]*Shanghai Jiao Tong University, Shanghai, 200240, China,*

Our laboratory focuses on development and application of methods for determination of the detailed structures of glycans and glycoconjugates. We explore the range of ExD approaches for achieving these goals. We make extensive use of a SolariX FT-ICR MS for mechanistic studies and to obtain data that underlie our *de novo* approach to interpretation of the complex ExD spectra of glycans, a Fusion Lumos Tribrid Orbitrap MS with ETD for extracting highly reliable data on the nLC-MS/MS timescale, gTIMS-FTICR and a 6560 qTOF MS modified with an ExD cell for resolution on the basis of shape, and a Hybrid QE-HF Orbitrap MS combined with an Omnitrap to explore the effects of accessing a wide range of electron energies. The results from the several instrument configurations are complementary and the observed fragmentation pathways are consistent across the platforms. This gives assurance that the methods for data acquisition and spectral interpretation can confidently be applied in various laboratories. As expected, the sensitivity and time required for spectral acquisition do vary substantially. Recent applications that resulted in site-specific assignments of glycopeptiforms are used as examples, including investigations of high mannose structures and the intact glycopeptiforms, binding mechanisms of wt and variant SARS-CoV-2 spike protein and alternative receptors, glycosylation changes in $\alpha 2\beta 1$ integrins involved in mutation-induced Primary Myelofibrosis, and determinations of glycosphingolipids.

(P071) Terminal α1-2 Fucosyl glycoconjugates synthesized by FUT1: A novel key regulator function in cell-fate decision during early development

Tair Swissa[1], Saray Chen[1,2], Michal Nagar[1], Liraz Efrat Shachar[1], Rachel G. Lichtenstein[1,2]

[1]*Avram and Stella Goren-Goldstein Department of Biotechnology Engineering, Faculty of Engineering Sciences,* [2]*Regenerative Medicine and Stem Cell (RMSC) Research Center, Ben Gurion University of the Negev, Be'er Sheva 8410500, Israel,*

Embryonic cell surface is rich in glycosphingolipids (GSLs), which their subsets can vary during differentiation. However, the reasons for GSL subset alteration during early embryogenesis remain elusive. By combining genomic approaches, flow cytometry, confocal imaging and transcriptomic data, we find that α1-2 fucosylated GSLs control differentiation of human pluripotent cells (hPCs) to tissues of the germ layers. We show that over-expression of α1-2 fucosylated GSLs disrupts hPC differentiation to mesodermal and endodermal cell lineages and attenuates cell differentiation potential to give rise to cardiomyocytes, while silencing α1-2 fucosylated GSLs prompt hPC differentiation and mesoderm commitment, when cells are exposed to external signals. We find the bone morphogenetic protein 4 (BMP4), a mesodermal gene inducer, as a suppressor factor of α1-2 fucosylated GSL expression. Conversely, over-expression of α1-2 fucosylated GSLs dampens many of early mesodermal genes, specifically impairs SMAD activation, despite BMP4 presence. An absence of α1-2 fucosylated GSLs in early/late mesoderm and primitive streak of mouse embryos is found, which is consistent with the results of human cultures. These findings identify a subset of α1-2 fucosylated GSLs, which may regulate early cell-fate decision and embryo development through modulation of cell signaling.

(P072) Role of proteoglycans in determining muscle stem cell fate during pregnancy

Francesca Boscolo Sesillo[1], John B. Rudell[1], Pamela Duran[1], Kirk Hansen[2], Karen L. Christman[1], Adam Engler[1], Marianna Alperin[1]

[1]*Department of Obstetrics, Gynecology, and Reproductive Sciences, Division of Urogynecology and Pelvic Reconstructive Surgery, University of California, San Diego, La Jolla, CA, 92037, USA,* [2]*Sanford Consortium for Regenerative Medicine, La Jolla, CA, 92037, USA,* [3]*Shu Chien-Gene Lay Department of Bioengineering, University of California, San Diego, La Jolla, CA, 92093, USA,* [4]*Department of Biochemistry and Molecular Genetics, University of Colorado, Aurora, CO, 80045, USA*

Pelvic floor disorders (PFDs) are prevalent and costly conditions that disproportionally impact women. Pelvic floor muscle (PFM) dysfunction consequent to maternal birth injury is a key risk factor for PFDs. Using the validated rat model, we have

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shown that during pregnancy, PFMs undergo longitudinal muscle growth via sarcomerogenesis, which protects PFMs from injury associated with strains during parturition. Muscle stem cells (MuSCs) are known regulators of skeletal muscle plasticity in response to various physiological cues. Content and properties of the MuSC niche that is mainly comprised of extracellular matrix (ECM) greatly impacts MuSC fate. To ultimately understand the mechanisms governing pregnancy-induced protective PFM adaptations, we aimed to elucidate the impact of pregnancy on pelvic MuSC fate. Using robust markers for proliferating and differentiated MuSCs, we identified significant increase in proliferation and differentiation of MuSCs isolated from PFMs of mid- (MP) compared to non- (NP) and late-pregnant (LP) animals. To determine potential mechanisms accountable for the MuSC phenotype observed in MP state, we interrogated PFM ECM composition using high-resolution proteomics and disaccharide analysis for heparan sulfate (HS) and chondroitin sulfate (CS). Proteomic analysis revealed significant increase in PFMs' small leucine-rich proteoglycans, specifically fibromodulin, decorin, and lumican, in MP relative to NP and LP states. Disaccharide analysis showed no difference in HS throughout pregnancy, but a significant increase in CS content and sulfation (4-O sulfation specifically) in PFM ECM in late pregnancy. Using FACS and RT-PCR, we then determined that the major source of the proteoglycan is fibroadipogenic progenitors (FAPs), known for their important role in aiding MuSC progression through myogenic lineage. In addition, while the expression of genes coding for these proteoglycans was similar in FAPs isolated from the PFMs of NP and MP rats, it was significantly decreased in FAPS isolated from the PFMs of LP animals. Our experimental results, combined with the existing literature, led us to the following theoretical framework: high proteoglycan content of the PFM ECM in mid-pregnancy promotes MuSC differentiation through sequestration of factors known to inhibit MuSC differentiation, such as myostatin. In late pregnancy, increased CS changes PFM stiffness, driving MuSCs back into quiescence. We are testing these hypotheses in our ongoing studies through 1) glycan arrays to test interactions between proteoglycans and various factors, known to impact MuSC differentiation and 2) mechanical testing of muscle stiffness. Understanding what governs protective pregnancy-induced adaptations of PFMs – integral to the proper function of the female pelvic floor- is vital for the development of scientifically-rationale preventative strategies for PFDs.

(P073) O-GlcNAcylation regulates OTX2's aggregation and stability

Eugenia Wulff-Fuentes[1], Jeffrey Boakye[2], Kaeley Kroenke[1], Rex R. Berendt[1], Carla Martinez-Morant[1], Michaela Pereckas[1], John A. Hanover[2], Stephanie Olivier-Van Stichelen[1,3]

[1]*Department of Biochemistry, Medical College of Wisconsin, Milwaukee WI, USA,* [2]*Laboratory of Cell and Molecular Biology, NIDDK, National Institutes of Health, Bethesda MD, USA,* [3]*Department of Obstetrics and Gynecology, Medical College of Wisconsin, Milwaukee WI, USA,*

O-GlcNAcylation is a key post-translational modification, playing a vital role in cell signaling during development, especially in the brain. In this study, we investigated the role of *O*-GlcNAcylation in regulating the homeobox protein OTX2, which contributes to various brain disorders, such as combined pituitary hormone deficiency, retinopathy, and medulloblastoma.

Our research demonstrated that, under normal physiological conditions, the proteasome plays a pivotal role in breaking down endogenous OTX2. However, when the levels of OTX2 rise, it forms oligomers and/or aggregates that require macroautophagy for clearance. Intriguingly, we demonstrated that *O*-GlcNAcylation contributes to enhancing the solubility of OTX2, thereby limiting the formation of these aggregates. Additionally, we unveiled a novel interaction between OTX2 and the chaperone protein CCT5 at the *O*-GlcNAc sites, suggesting a potential collaborative role in preventing OTX2 aggregation. Finally, our study demonstrated that while OTX2 normally promotes cell proliferation, an O-GlcNAc-depleted OTX2 triggers cytotoxic effects.

These findings suggest that the aggregation and autophagic degradation of OTX2 serve as a protective mechanism against aberrant OTX2 expression, as observed in medulloblastoma. However, in the context of cancer, marked by hyper-*O*-GlcNAcylation, this mechanism could lead to OTX2 stabilization, potentially contributing to cancer progression.

(P074) A detailed enzymatic description of the Arabidopsis FRIABLE1 Rhamnogalacturonan-I Rhamnosyltransferase

Kayleigh Robichaux[1], Robert Amos[2], Camryn Lee[1], Debra Mohnen[2], Ian Wallace[1,2]

[1]*Department of Biochemistry and Molecular Biology, University of Nevada, Reno, Reno, NV 89557 USA,* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens,*

Plant cell wall biosynthesis requires the coordination of numerous glycosyltransferases for the synthesis of cell wall resident polysaccharides, including cellulose, hemicelluloses, and pectins. Pectins are structurally complex plant cell wall polymers that contain a characteristically high galacturonic acid content, and these polymers can be functionally subdivided into Homogalacturonan (HG), Rhamnogalacturonan-I (RG-I), and Rhamnogalacturonan-II (RG-II) domains. RG-I is a complex polysaccharide consisting of a repeating rhamnose (α 1→4)-galacturonic acid (α 1→2) disaccharide backbone that can be further

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elaborated with arabinan or galactan side chains. Recently, a family of GT106 genes were described as Rhamnogalacturonan-I:Rhamnosyltransferases (RRTs), which catalyze the rhamnosylation of the RG-I (d-GalA-l-Rha)$_{(n)}$ repeating backbone. However, a detailed characterization of purified RRT enzymes has not been completed. Here, we performed a thorough biochemical analysis of the *Arabidopsis thaliana* FRIABLE1 (FRB1)/ RRT8 protein. RRT8 was robustly active upon purification and catalyzed the mono-rhamnosylation of purified RG-I oligosaccharides terminating in galacturonic acid. The Arabidopsis RRT family contains 10 isoforms, and a comparison between RRT8 and closely related AtRRT1 and AtRRT9 revealed that RRT8 is 10-times more active than other Arabidopsis RRTs. Site-directed mutagenesis studies based on foundational functional knowledge from *C. elegans* protein *O*-fucosyltransferase I X-ray structure and AlphaFold models revealed amino acid residues that are critical for catalysis and suggest a role for the N-terminal "stem" domain in controlling RRT8 activity. These results provide a strong mechanistic understanding of RRT8 as a representative of the GT106 family and further highlight activity heterogeneities within the Arabidopsis GT106 family which may be relevant to glycan structural diversity during RG-I biosynthesis in different plant tissues.

(P075) Roles of glypican and heparan sulfate modifying enzymes in synaptic plasticity at the Drosophila neuromuscular junction

Keisuke Kamimura[1], Aiko Odajima[1], Hiroshi Nakato[1], Chiaki Ohtaka-Maruyama[1]

[1]*Developmental Neuroscience Project, Department of Brain and Neurosciences, Tokyo Metropolitan Institute of Medical Science,*

Heparan sulfate proteoglycans (HSPGs) are involved in various organ development, including the nervous system, but their role in synaptic plasticity is poorly understood. In this study, we have investigated the roles of HSPG and heparan sulfate (HS) modification in synaptic plasticity using the fruit fly, *Drosophila,* neuromuscular junction (NMJ), a model of glutamatergic synapses. In *Drosophila*, starvation causes increases in synaptic terminals in the NMJ and the migration speed of larvae. Our previous studies have shown that HSPG glypican, Dally-like (Dlp), is required for these starvation-dependent morphological and behavioral plastic changes. Therefore, we next investigated the roles of heparan sulfate (HS) chains of Dlp and its modification in these processes. We used CRISPR/Cas9-mediated genome editing to generate $dlp^{\Delta HS}$ flies in which HS chains were deleted by inserting mutations into the HS attachment sites of Dlp core protein. We found that starvation did not increase the number of synaptic terminals and the migration speed of $dlp^{\Delta HS}$ larvae, indicating that HS chains are required for Dlp function in starvation-mediated synaptic and behavioral plasticity. Next, we investigated the possibility that the fine structures of HS chains regulate synaptic plasticity using flies lacking HS 3-*O* sulfotransferase B (*Hs3st-B*) and 6-*O* endosulfatase (*Sulf1*). Significantly, either *Hs3st-B* or *Sulf1* loss inhibited the starvation-dependent formation of synaptic terminals. The increase in synaptic terminals during starvation requires an increase in GluRIIA, one of the glutamate receptor subunits, in the postsynaptic region. We found that deletion of either *Hs3st-B* or *Sulf1* did not cause a starvation-dependent increase of GluRIIA. These results suggest that Dlp induces starvation-dependent synaptic plasticity by regulating the levels of GluRIIA through the fine structures of the HS chains.

(P076) Identification of Physiological Lectin Receptor Ligand Repertoires in Health and Disease

Mayank Saraswat[1], John Hintze[1], Damien Restagno[1], Jamey D. Marth[1]

[1]*Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, 92037,*

The prototype mammalian lectin, the Ashwell-Morell receptor (AMR) was first discovered and isolated almost 50 years ago. Since then, dozens of lectins spanning multiple molecular families have been identified in the mammalian genome. However, their physiological ligands and biological functions have mostly eluded our understanding. Difficulties in identifying physiological ligands of lectin receptors have reflected in large part the methods commonly used in proteomic analyses, often during which glycoproteins are denatured and aggregated by laboratory techniques including electrophoresis and surface binding. The resulting presentation of high glycan densities is in contrast with lectin binding studies applied instead to non-denatured glycoproteins in solution by using lectin affinity chromatography. This later approach allowed the first look at physiological ligands regulated by AMR function among the blood proteome. We have therefore been applying non-denaturing lectin affinity chromatography of blood plasma from mice and humans in contexts of health and disease including sepsis to identify lectin ligand repertoires in the blood proteome and their changes linked to physiological outcomes. We are applying both plant lectins and animal lectins to compare ligand repertoires among mammalian blood proteins, with results thus far consistent with significant ligand overlap. Following comparative mass spectrometry, the identification of candidate physiological ligands can be further validated using immunological and biochemical techniques. Using this approach, we have determined that, at steady state, mammalian lectin receptors each uniquely modulate subsets of blood glycoprotein ligands and thus far each lectin has a ligand repertoire

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of between 0.3%-10% of blood proteome mass and from approximately 1%-10% of proteome diversity. This translates to each lectin receptor studied thus far exhibiting a unique ligand repertoire of between 50–500 blood glycoproteins, with no significant overlap yet other than that found among the Asgr1 and Asgr2 subunits of the AMR. We have previously identified blood glycoprotein ligands of the Asgr1 and Asgr2 components of the AMR and have more recently identified blood glycoprotein ligands of the alpha-M Integrin lectin and the Mrc1 lectin. Meta-analysis of the data spanning biological processes and pathways linked to these lectin receptors have so far successfully predicted the presence of previously undetected phenotypes in lectin receptor deficient mice.

(P077) Chemoenzymatic synthesis of fucosylated and/or sialylated linear human milk oligosaccharides (HMOs) with a hexaose core

Zimin Zheng[1], Anand Kumar Agrahari[1], Yuanyuan Bai[1], Hai Yu[1], Xi Chen[1]

[1]*University of California, Davis,*

Human milk oligosaccharides (HMOs) constitute a major component of human milk. There is an increasing attention on the contribution of HMOs to the health of breast-fed infants. They are not digested by infants but instead found in their guts, urine and plasma, which can serve as prebiotics to suppress the growth of some pathogenic bacteria, anti-adhesive decoy receptors for some pathogenic microbes, antibiofilm antimicrobials, brain-gut axis modulators, immune modulators, infant colon epithelial cell response modulators, and cell maturation stimulators. The detailed functions of specific HMOs, especially those with more complex structures, are not clear. Exploring the applications of HMOs as infant formula additives, nutraceuticals, and/or therapeutics has begun but has been slow due to the limited access to structurally defined HMOs in sufficient amounts. I have contributed to the development of a highly efficient user-friendly glycosyltransferase-based synthetic platform to access target HMOs in a systematic manner. I have been working on developing efficient routes for systematic chemoenzymatic synthesis of HMOs containing a hexose core (pLNnH, pLNH) using various approaches including Enzyme Assembly Synthetic Maps (EASyMaps), Stepwise One-Pot Multienzyme (StOPMe) with *in situ*-generation of sugar nucleotides, glycosyltransferase substrate engineering strategy, and glycosyltransferase engineering, etc. The products are essential probes and reagents for elucidating the roles of HMOs and exploring their applications. The chemoenzymatic synthetic process is readily scalable for large-scale production of HMOs in the future.

(P078) Unraveling the Heparan Sulfate Proteoglycan FGF1 Axis in Organismal Energy Metabolism

Ariane Pessentheiner[1], Chung-Jui Yu[1], Marissa Maciej-Hulme[1], Nathan Thomas[1], Shihao Liu[2], Greg Trieger[3], Michael Downes[2], Ronald M. Evans[2], Kamil Godula[3,4], Philip Gordts[1]

[1]*Division of Endocrinology and Metabolism, School of Medicine, University of California, San Diego,* [2]*Salk Institute, Gene Expression Laboratory, La Jolla, CA,* [3]*Department of Chemistry and Biochemistry, University of California, San Diego,* [4]*Glycobiology Research and Training Center, University of California, San Diego*

Insulin resistance is a critical factor underlying numerous metabolic disorders, including obesity, diabetes, and cardiovascular disease. Within this context, adipocytes and their extracellular matrix actively contribute to metabolic adaptations that arise during excessive nutrient intake, thereby driving the emergence of insulin resistance. Heparan sulfate proteoglycans (HSPGs) govern many biological processes, including adipocyte biology. However, the precise physiological significance of adipocyte HSPGs remains largely elusive. In this study, we leveraged adipose tissue-specific knock-out models of N-acetylglucosamine-N-deacetylase-N-sulfotransferase 1 (*Ndst1* AKO) and Exostosin 1 (*Ext1* AKO) to investigate the central role of HSPGs in regulating adipocyte homeostasis during nutrient excess. Our findings demonstrated that HSPG loss and under-sulfation profoundly impact systemic glucose homeostasis via fibroblast growth factor 1 (FGF1) signaling. Remarkably, both *Ext1* AKO and *Ndst1* AKO mice exhibited heightened weight gain compared to their wild-type counterparts when subjected to a high-fat diet, along with the development of significant diabetic features, including glucose intolerance, insulin resistance, and fatty liver. Previous studies have highlighted the potent insulin-sensitizing properties of HS-dependent FGF1, which holds promising therapeutic potential. However, our results revealed the absence of FGF1-mediated insulin sensitization in *Ext1* AKO and *Ndst1* AKO mice. In addition, compound inactivation of *Ndst1* and *Fgfr1,* thereby neutralizing FGF1 signaling in adipocytes in mice, resulted in no additive metabolic phenotype due to loss of HS sulfation establishing that adipocyte HSPGs require FGF1 signaling to control glucose metabolism. Our findings provide compelling support for the notion that natural variation in HSPG composition function as a critical regulator, fine-tuning adipocyte function and serving as predictive markers for susceptibility to insulin resistance, type-2 diabetes, and other metabolic complications arising from obesity.

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(P079) Got Milk (Oligosaccharides)? A Multi-Glycomic Structural Workflow for Milk Samples

Lauren Pepi[1], Christopher Ashwood[1,2,3], Jamie Heimburg-Molinaro[1,2], Richard D. Cummings[1,2]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Boston, MA, USA,* [2]*Harvard Medical School, Boston, MA, USA,* [3]*Protea Glycosciences, Sydney, New South Wales, Australia,*

Human milk oligosaccharides (HMOs) are attributed to the high nutritional value human milk holds for newborns compared to bovine milk. Non-lactose free oligosaccharides are the third most abundant component of human milk, behind lactose and lipids. Multiple studies have focused on characterizing HMOs to better understand their function in infant growth and health. However, the heavy focus on HMOs ignores the remainder of the glycome. Herein we propose a multi-glycomics structure-specific approach for milk glycome analysis. This approach allows for the extraction and analysis of *N*-glycans, free oligosaccharides and glycans released from glycolipids. A library of over 200 glycan structures, including an average of 3 isomers for each composition, aided in determining structure-specific diagnostic fragments used to develop this method.

Human milk, bovine milk, and two forms of baby formula were used to validate and benchmark the method. Baby formula 1 (BF-1), contains 1 added HMO, whereas baby formula 5 (BF-5), contains 5 added HMOs. Though both formulas contain the added HMOs, they also contain bovine skim milk. To better assess the quantitative reproducibility of this method, matrix-matched calibration curves covering four orders of magnitude were constructed. We identified milk oligosaccharides unique to human in the human milk sample, and unique to bovine in the bovine milk and formula samples. Our results support that two of the 17 identified free oligosaccharides are specific for human, and all CV (coefficient of variation) values were less than 20% over three orders of magnitude, making this method suitable for sensitive quantitation. Three glycans from glycolipids were identified as unique to bovine milk. *N*-glycan quantitation was more variable, which may be attributed to the enzymatic release. Our multi-glycomics pipeline repeatedly quantifies oligosaccharides in three different flavors from a single milk sample, enabling quantitative measurements to be mapped back to their respective biosynthetic pathways for translational advancement.

(P080) Environmentally responsive *O*-GlcNAcylation Controls Stem/Progenitor Cell Homeostasis and DNA Damage Repair Signaling

John Hanover[1], Hyun-jin Na[1], Ilhan Akan[1], Lara Abramowitz[1]

[1]*Laboratory of Cell and Molecular Biology, NIDDK, National Inst. of Health,*

Using Drosophila midgut stem cells, mouse ES cells (mESCs) and mouse embryonic fibroblasts (MEFs), we examined stem cell self-renewal in O-GlcNAcase knockout models. These studies provide rigorous *in vivo* and *in vitro* evidence that interference with stress- or nutrient-responsive O-GlcNAc cycling induces proliferation and the DNA damage response pathway in stem cells via an autoregulatory loop. The model that emerges provides a paradigm-altering view of the means by which stem cells respond to nutrient flux and provides a mechanistic link between nutrient signaling, cell cycle regulation and the DNA damage response. After genetic and pharmacological dissection of the pathway, we discovered that the activity of both DDR sensors (ATM/ATR) and effectors (CHK1/CHK2) are required to maintain OGT protein stability. Thus, these *in vivo* findings provide evidence for an autoregulatory mechanism involving O-GlcNAc cycling and DDR signaling. Elevated stress- and nutrient-driven O- GlcNAcylation induces DDR and cell cycle check points which, in turn, alter OGT stability to modulate O-GlcNAcylation.

Importantly, the essential features of this pathway were conserved in mESCs and MEFs. Thus, our findings reveal a conserved mechanistic link between O-GlcNAc cycling, stem cell self-renewal, and DDR with profound implications for stem cell-derived diseases including cancer. Our published work has demonstrated that imbalanced O-GlcNAc cycling leads to a complex phenotype including an altered histone code, induction of DNA damage and repair pathways. We are now investigating the mechanism by which this genome instability may occur. We found that O-GlcNAcase deficient mouse ESCs or MEFs exhibit genome-wide changes in *de novo* DNA methylation. The hypomethylated genes and promoters include many imprinted genes. We determined that this global change in methylation is linked to deregulation of ~3000 transcripts including microRNAs and genes involved in development and differentiation. In a related study, we examined the coordination between a cytosolic N-glycanase, NGLY1, and O-GlcNAc, to maintain stem cell proliferation. Our data showed O-GlcNAcylation and NGLY1 have key roles in both progenitor and differentiated cells that contribute to tissue homeostasis. Furthermore, the NRF2 antioxidant signaling pathway and ENGase, an enzyme involved in the processing of free oligosaccharides in the cytosol, play key roles in this pathway through regulation of protein aggregation to contribute to gut maintenance. Taken together, these findings suggest that that alterations in O-GlcNAc cycling and NGLY1 could be future therapeutic targets.

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(P081) Innate Mechanism of Mucosal Barrier Erosion in the Pathogenesis of Acquired Colitis

Won Ho Yang[1,2], Peter V. Aziz[1], Douglas M. Heithoff[3], Yeolhoe Kim[2], Jeong Yeon Ko[2], Jin Won Cho[2], Michael J. Mahan[3], Markus Sperandio[4], Jamey D. Marth[1]

[1]Sanford-Burnham-Prebys Medical Discovery Institute, Infectious and Inflammatory Diseases Center, La Jolla, CA 92037. USA, [2]Glycosylation Network Research Center and Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea, [3]Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, CA 93106, [4]Walter Brendel Center for Experimental Medicine, Institute of Cardiovascular Physiology and Pathophysiology, Ludwig Maximilians University, Munich, Germany

The colonic mucosal barrier protects against infection, inflammation, and tissue ulceration. Composed primarily of Mucin-2, proteolytic erosion of this barrier is an invariant feature of colitis however the molecular mechanisms are not well understood. We have applied a recurrent food poisoning model of acquired Inflammatory Bowel Disease using *Salmonella enterica* Typhimurium to investigate mucosal barrier erosion. Our findings reveal an innate Toll-like receptor 4-dependent mechanism activated by previous infection that induces Neu3 neuraminidase among colonic epithelial cells concurrent with increased Cathepsin-G protease secretion by Paneth cells. These anatomically separated host responses merge with the desialylation of nascent colonic Mucin-2 by Neu3 rendering the mucosal barrier susceptible to increased proteolytic breakdown by Cathepsin-G. Depletion of Cathepsin-G or Neu3 function using pharmacological inhibitors or genetic-null alleles protected against Mucin-2 proteolysis and barrier erosion coincident with reduced the frequency and severity of colitis, revealing approaches to preserve and potentially restore the mucosal barrier.

(P082) Halofuginone-mediated repression of heparan sulfate limits hepcidin and alters iron metabolism

Stephanie Leal[1,2], Andrea Denardo[3], Maura Poli[3], Ralph Mazitschek[4], Jeffrey D. Esko[5], Philip L.S.M. Gordts[1,6]

[1]Department of Medicine, Division of Endocrinology and Metabolism, University of California San Diego, La Jolla, CA, USA, [2]Department of Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, California, CA, USA, [3]Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy, [4]Center for Systems Biology, Massachusetts General Hospital, Boston, MA, USA, [5]Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, USA, [6]Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA, USA,

Hepcidin is a liver-derived hormone known as the master regulator of iron metabolism. Thus far, three canonical pathways of hepcidin regulation are widely accepted, and they include the inflammatory pathway, where IL6 triggers the JAK/STAT pathway and stimulates hepcidin expression; the hyperferremia pathway, where high levels of iron cause sinusoidal cells in the liver to release BMP6 which stimulates hepcidin expression through the BMPR/SMAD pathway; and the erythropoiesis pathway, where EPO decreases hepcidin expression thereby increasing iron availability for red blood cell production. Previously, we reported a novel hepcidin regulatory pathway involving liver heparan sulfate proteoglycans (HSPGs). HSPGs are proline-rich transmembrane proteins decorated with extended HS chains that create negatively charged domains. The amount and degree of HS sulfation often modulate interactions between growth factors and receptors. Recently, we discovered that halofuginone reduces HS biosynthesis. Halofuginone, an anti-fibrotic agent, is an analog of febrifugine, an alkaloid found in the plant *Dichroa febrifuga*. It acts competitively with proline by binding the prolyl tRNA synthase (PRS) active site of the human glutamyl-prolyl tRNA synthetase, limiting proline utilization during protein translation. Many HS biosynthesis enzymes and HS proteoglycan core proteins are proline-rich, which explains their susceptibility to halofuginone's translational suppression. However, it is unknown whether the FDA-approved small molecule halofuginone can be harnessed to dampen hepcidin expression. We report that halofuginone can limit hepcidin expression in a hyperferremic mouse model (8.3 g/kg carbonyl iron), after 1- and 3-weeks of treatment, and reduce hyperferremia. This effect was not observed in mice presenting with impaired liver heparan sulfate sulfation (*Ndst1$^{f/f}$Alb-Cre^{+}*), confirming that halofuginone targets the HSPG-mediated hepcidin activation. However, halofuginone did not alter hepcidin levels in mice experiencing acute inflammation (LPS 0.1 mg/kg, 6 h). Our in-vitro studies in human hepatoma cells (Hep3B and HepG2) show that halofuginone treatment inhibits basal and BMP6-induced hepcidin levels in a dose and time-dependent fashion. Like our in-vivo results, hepatoma cells lacking HS polymerization (*EXT1$^{-/-}$* Hep3B) halofuginone did not affect basal and BMP6-induced hepcidin expression. Together, our studies demonstrate that halofuginone can be used to treat iron-restricted anemic disorders characterized by high levels of hepcidin and reveal halofuginone as a putative therapeutic compound.

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(P083) Engineering Homogenous Glycoforms of Blood Glycoproteins for Discovering Physiological Ligand Determinants of the Ashwell-Morell Receptor

John Hintze[1], Robert Fraumeni[1], Noortje De Haan[2], Zhang Yang[2], Henrik Clausen[2], Jamey D. Marth[1]

[1]*Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, 92037,* [2]*Copenhagen Center for Glycomics, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark,*

Mechanisms determining the half-lives of blood proteins have been difficult to perceive. In the simplest case, the rate of elimination from the bloodstream is determined by proteinaceous receptor-ligand binding and endocytic uptake. However, most proteins present in the blood do not have corresponding endocytic receptors that recognize their protein sequences. That would require many hundreds if not thousands of dedicated clearance receptors, and for the vast majority of circulating blood proteins, that does not appear to be the case. Instead, most secreted proteins are post-translationally modified with N-glycans, which when remodeled by glycosidases at different rates in circulation can then be recognized by endocytic lectin receptors expressed among the vasculature and parenchyma of multiple organs. The two most abundant N-glycan termini of blood glycoproteins are sialic acid linked to galactose in α2-3 or α2-6 linkage. Endogenous neuraminidases in the blood can remove one or both types of sialic acid thereby unmasking underlying galactose linkages that when presented as tri-valent or higher become preferred ligands of the prototypical endocytic lectin the Ashwell-Morell receptor (AMR) of the liver hepatocyte. Whether functional glycoproteins bearing either Siaα2-3Gal or Siaα2-6Gal glycan termini exhibit different rates of desialylation and engagement with the AMR has been approached primarily using synthetic neoglyconjugates. Here we use glycoengineered CHO cells to produce two different human alkaline phosphatase isozymes (IAP and TNAP) both fully N-glycosylated and carrying only bi-antennary N-glycans capped with either α2-3 or α2-6 linkages of sialic acid (Neu5Ac). We are now comparing the circulatory half-lives of these glycoprotein enzymes in vivo among wild-type mice as well as littermates lacking the Asgr1 or Asgr2 protein subunit of the AMR. Data analyzed thus far indicates that neither sialylated termini Siaα2-3Gal or Siaα2-6Gal are ligands of the AMR, unlike their desialylated glycoprotein preparations. The impact of these sialic acid linkages and their rates of hydrolysis in AMR-dependent clearance, are being investigated. Our findings will reveal key determinants in the production of physiological AMR ligands in vivo among circulating blood glycoproteins.

(P084) Receptor protein tyrosine phosphatases are required for sensory axon wiring and regulated by POMT-mediated O-mannosylation in *Drosophila*

Pedro Monagas-Valentin[1], Robert Bridger[2], Ishita Chandel[1], Boris Novikov[1], Lance Wells[2], Vladislav Panin[1]

[1]*Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA,* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA,*

Protein O-mannosylation is a type of post-translational modification necessary for neuromuscular development in animals. Mutations in Protein O-mannosyltransferases 1 and 2 (POMT1/2) result in a group of severe congenital muscular dystrophies associated with defective glycosylation of α-Dystroglycan (α-Dg) termed dystroglycanopathies. Although dystroglycanopathies are known to include prominent neurological phenotypes, the pathomechanisms of these defects remain poorly understood. POMT1/2 substrates other than Dg are thought to play important functions in the nervous system, but they remain largely uncharacterized. Our experiments have shown that *Dg* alone does not account for *POMT1/2* phenotypes in *Drosophila*, indicating the involvement of other substrates in the POMT-mediated pathway. Using a variety of genetic approaches in *Drosophila*, we identified *Receptor Protein Tyrosine Phosphatase 69D* (*PTP69D*) as a gene interacting with *POMT1/2* in the pathway required for sensory axon connections in the larval ventral ganglion. These interactions can be synergistic or antagonistic depending on the mode of genetic manipulation. Using glycoproteomic approaches, we found that PTP69D is an *in vivo* substrate for POMT1/2. To further elucidate the role of O-mannosylation in regulating RPTPs, our ongoing work focuses on the analyses of phosphatase activity in a variety of POMT1/2 and RPTP genetic backgrounds. We will present new results suggesting that O-mannosylation is essential for the proper level of phosphorylation in the *Drosophila* nervous system. These mechanisms are potentially conserved in mammals and may shed light on the involvement of POMT1/2 and RPTPs in human pathologies. This project was supported in part by grants from NIH (NS099409 & NS075534 to V.P., and GM111939 to L.W.), CONACYT (2012-037S) and the Texas A&M AgriLife Institute for Advancing Health Through Agriculture to V.P.

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(P085) Developmental and organ-specific expression profiles of sulfated sialic acids in medaka fish as revealed by the chemical method

Sota Yamashita[1,2], Di Wu[1,2,3], Masaya Hane[1,2,3], Naoko Komura[4], Hiromune Ando[4], Chihiro Sato[1,2,3], Ken Kitajima[1,2,3]

[1]*Grad. Sch. Bioagr. Sci, Nagoya Univ., Japan,* [2]*Biosci. Biotech. Center, Nagoya Univ., Japan,* [3]*iGCORE, Nagoya Univ., Japan,* [4]*iGCORE, Gifu Univ., Japan*

Sialic acids (Sias) modify cell surface and extracellular glycoproteins and glycolipids and play important roles in cell-to-cell recognition and signal transduction. Sias are known to undergo various modifications including *O*-acetylation, *O*-sulfation, and *O*-methylation. However, nothing has been known about responsible enzymes and biological functions of these modifications except for *O*-acetylation. We have been focusing on *O*-sulfation, and found that, in sea urchin gametes, 8-*O*-sulfated *N*-acetyl and *N*-glycolylneuraminic acid (Neu5Ac8S and Neu5Gc8S) occurred in gangliosides, being involved in the sperm-egg interaction at fertilization. Although Neu5Ac8S and Neu5Gc8S are reported in gangliosides in bovine gastric mucosa, no functional study has been done in vertebrate so far. In 2022, our laboratory first identified Wscd1 and Wscd2 as the sialate:*O*-sulfotransferases (SulT-Sia) in mouse, human, and medaka fish, and this finding has allowed us to elucidate biological functions of sulfated sialic acids (SiaSs). Our objective is to reveal biological significance of the SiaS in vertebrates. For this purpose, not only analysis of Wscd1- and Wscd2-knockout (KO) animals, but also development of chemical and immunochemical methods to detect SiaSs are important. Thus, this study sought to investigate the occurrence and amount of SiaS in medaka (*Oryzias latipes*) using the following chemical and immunochemical methods. First, Embryos and larva at 0–15 days post-fertilization (dpf) and various organs from 3-month-old medaka were prepared and analyzed by the fluorometric high performance liquid chromatography (HPLC) analysis. The results showed that Neu5Ac9S was detected in embryos and larva, and that some adult organs contained both Neu5Ac8S and Neu5Ac9S, while others contained either Neu5Ac8S or Neu5Ac9S. It is suggested that Neu5Ac9S starts expressing at early developmental stages, while Neu5Ac8S appears in adult in an organ-specific manner. Second, *Wscd1*-KO and *Wscd2*-KO medaka were generated and the adult brain and testis were subjected to the fluorometric HPLC analysis. In brain, the amounts of Neu5Ac8S and Neu5Ac9S remained unchanged in both *Wscd1*-KO and *Wscd2*-KOs. In testis, Neu5Ac8S was lost in *Wscd2*-KO, while Neu5Ac9S was lost in both cases. Finally, fertilized eggs derived from WT pairs and *Wscd2*-KO pairs were observed during prehatching stage, and showed that, although there was no difference between WT and Wscd2-KO in survival rate, the hatching date of *Wscd2*-KO was delayed.

(P086) Role of Casd1-dependent sialic acid 9-O-acetylation in maintaining colon mucus function and homeostasis in vivo

Simin Jafaripour[1], Mackenzie Melvin[1, 2], Irving Zhang[1], Colin Parrish[3], Brian Wasik[3], Negin Kazemian[4], Hannah Olsson[1], Sepideh Pakpour[4], Wesley F. Zandberg[2], Kirk Bergstrom[1]

[1]*University of British Columbia (Okanagan), Department of Biology,* [2]*University of British Columbia (Okanagan), Department of Chemistry,* [3]*Cornell University, College of Veterinary Medicine, Ithaca, New York, USA,* [4]*University of British Columbia (Okanagan), School of Engineering*

The *O*-glycosylated intestinal mucus network—made up of polymers of the MUC2 mucin— provides innate immune defence to protect our gastrointestinal tract from microbial insult. Sialic acid (Sia) is a key capping monosaccharide on complex *O*-glycans which has recently been linked to preserving mucus integrity. Sia is extensively modified by *O*-acetyl (*O*Ac) groups at the C-4, -7, -8, and/or -9 positions. However, the extent and role of these *O*Ac modifications in human and murine mucus is unclear. We hypothesized that Sia's protective functions on gut mucus are regulated through its *O*-acetylation. To address this, we first used viral-derived probes that target specific *O*Ac-Sia analogues in mucus on sections from human and mouse feces and colon tissues, finding *O*Ac-Sia was distributed throughout the niche and barrier layers of mucus in humans and mice in situ. We next analyzed the sialome on glycans derived from purified human fecal MUC2 and mouse Muc2 where *O*Ac-Sia analogues were quantitated by HPLC-MS after derivatization with 4,5-dimethyl-1,2-diaminobenzamine (DMBA). We found Sia on both human MUC2 and murine Muc2 were heavily *O*-acetylated, with ~75% and ~45% of Sia having various combinations of 4, 7, 8, and/or 9-*O*Ac-based Sia modification in humans and mice respectively. 9-*O*-acetylation of Sia is mediated by the sialate *O*-acetyltransferase CAS1 domain containing 1 (CASD1). Therefore, to understand the biologic roles of *O*Ac-Sia in vivo, we generated intestinal epithelial cell-specific Casd1 KO mice (*Casd1flox/flox*;VillinCre or IEC *Casd1-/-* mice) and analyzed their mucins and phenotypes vs. WT littermates. IEC *Casd1-/-* mice were viable and healthy with knockdown confirmed by lack of 9-*O*Ac signals on sections and via western blot of protein lysates and mucins. Notably, sialomics on extracted Muc2 showed loss of gut epithelial Casd1 impaired *O*-acetylation at the C-7, -8, and -9 positions. Consistent with the known role of *O*Ac Sia in sialidase inhibition, loss of *O*Ac Sia was associated with increased sialidase activities as assessed by heightened fluorogenic substrate 4-MU-Neu5Ac in fecal supernatants in IEC *Casd1-/-* vs. WT mice. Paradoxically, we observed increased Sia (Neu5Ac) on Muc2 from IEC *Casd1-/-* mice, possibly explained by a remodelling of the sialome, where *O*-glycomics revealed a unique population of sialylated *O*-glycans in the absence of Casd1 activity. This, in turn, could explain why mucus barrier function appeared intact regardless of 9-*O*Ac status. However, IEC *Casd1-/-* mice showed heightened susceptibility to 1.5% w/v Dextran

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Sodium Sulfate (DSS) colitis, linked to thinning of the mucus in IEC *Casd1-/-* vs. WT littermates after challenge. Taken together, this suggests 9-OAc via Casd1 governs the majority of OAc-Sia modifications on Muc2 in mice, and may be necessary for a fully adaptive sialome on mucus to mediate protection in response to mucosal challenge.

(P087) Mn007 restores O-mannosyl glycosylation of alpha dystroglycan in FCMD patient's models

Mariko Taniguchi-Ikeda[1], Michiyo Koyanagi-Aoi[2], Kenta Morita[3], Tatsuro Maruyama[3], Yoichiro Harada[4], Hidetoshi Sakurai[5], Takashi Aoi[2]

[1]*Department of Clinical Genetics, Fujita Health University, Japan,* [2]*Division of Stem Cell Medicine, Kobe University Graduate School of Medicine, Japan,* [3]*Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Japan,* [4]*Department of Glyco-Oncology and Medical Biochemistry, Osaka International Cancer Institute, Japan,* [5]*Department of Clinical cell Applications CiRA, Kyoto University, Japan,*

Fukuyama congenital muscular dystrophy (FCMD) is a severe, intractable genetic disease that affects the skeletal muscle, eyes, and brain and is attributed to a defect in alpha dystroglycan (αDG) O-mannosyl glycosylation. We previously established disease models of FCMD; however, they did not fully recapitulate the phenotypes observed in human patients. In this study, we generated induced pluripotent stem cells (iPSCs) from a human FCMD patient and differentiated these cells into three-dimensional brain organoids and skeletal muscle. The brain organoids successfully mimicked patient phenotypes not reliably reproduced by existing models, including decreased αDG glycosylation and abnormal radial glial cell (RG) fiber migration. The basic polycyclic compound Mannan-007 (Mn007) restored αDG glycosylation in the brain and muscle models tested and partially rescued the abnormal RG migration observed in cortical organoids. Therefore, our study underscores the importance of αDG O-mannosyl glycans for normal RG architecture and proper neuronal migration in corticogenesis.

(P088) Is the linkage mode of sialic acid residues significant in normal heart development in medaka?

Ken Kitajima[1], Takayuki Omoto[1], Di Wu[1,2], Emi Maruyama[1], Katsue Tajima[1], Masaya Hane[1,2], Chihiro SATO[1,2]

[1]*Nagoya University/Bioscience Biotechnology Center and Graduate School of Bioagricultural Sciences,* [2]*Nagoya University/Institute for Glyco-core Research (iGCORE),*

Sialic acids (Sias) are often linked to galactose (Gal) residues by α2,6- and α2,3-linkages in glycans of glycoproteins. Sias are indispensable for vertebrate development, because organisms deficient in some enzymes in the Sia synthetic pathway are lethal during the development. However, it remains unknown if the difference of Siaα2,6Gal or α2,3Gal linkage has a critical meaning during development. To find a clue to understand significance of the linkage difference at the organism level, medaka was used as a vertebrate model. In embryos, Siaα2,6Gal epitopes recognized by *Sambucus nigra* lectin (SNA) and Siaα2,3Gal epitopes recognized by *Maackia amurensis* lectin (MAA) were enriched in the blastodisc and the yolk sphere, respectively. When these lectins were injected in the perivitelline space, SNA, but not MAA, impaired the embryo body formation at 1 day post-fertilization (dpf). Most Siaα2,6Gal epitopes occurred on *N*-glycans due to their sensitivity to the peptide:*N*-glycanase F. Of knockout-medaka (KO) for either of two β-galactoside:α2,6-sialyltransferase genes, *ST6Gal I* and *ST6Gal II*, only *ST6Gal I*-KO showed severe cardiac abnormalities at 7–16 dpf, leading to lethality at 14–18 dpf. Interestingly, however, these cardiac abnormalities of *ST6Gal I*-KO were rescued by forced expression of *ST6Gal II* or the b-galactoside:α2,3-sialyltransferase IV gene (*ST3Gal IV*) as well as by that of *ST6Gal I*. These results indicate that the Siaα2,6Gal linkage synthesized by ST6Gal I are critical in heart development; however, it can be replaced by the linkages synthesized by ST6Gal II and ST3Gal IV. These data suggest that sialylation itself is more important than its particular linkage for the heart development.

(P089) O-GalNAc glycans enrich in white matter tracks of the mammalian brain

Maxence Noel[2], Suttipong Suttapitugsakul[2], Richard Cummings[2], Robert Mealer[1]

[1]*Department of Psychiatry, Oregon Health & Science University, Portland, OR,* [2]*National Center for Functional Glycomics, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA,*

Protein O-glycosylation are essential post-translational modifications involved in brain development and disease. Genetic studies associate several O-GalNAc glycosylation genes with neuropsychiatric phenotypes, in addition to a recently described congenital disorder of glycosylation with intellectual disability caused by mutations in C1GALT1C1. In drosophila, O-GalNAc glycans

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have been shown to be involved in brain development and synaptic function. However, there is limited information about O-GalNAc glycans in the mammalian brain, despite these structures being the most abundant type of O-glycan present based on our previous studies using MALDI-TOF MS glycomics. Here, we explore the cellular specificity, spatial distribution, and protein carriers of O-GalNAc glycans in mouse brain using multiple methods including cell-specific deletion, lectin binding, and glycoproteomics. Genetic ablation of O-GalNAc extension in neurons or astrocytes had minimal effect on total O-glycans levels and failed to replicate the severe neurologic phenotype seen in rodent models and human disorders of O-GalNAc synthesis. By examining their spatial distribution, we observed striking enrichment of O-GalNAc glycans in white matter tracts which was noticeably distinct from N-glycans. Glycoproteomic analysis demonstrated that O-GalNAc glycans are expressed by multiple cell types and present on proteins involved in brain development, synapse organization, and the extracellular matrix. These findings highlight novel aspects of O-GalNAc biology in the mammalian brain and will inform future studies of their role in human brain development and disease.

(P090) Hypothalamic perineuronal net sulfation patterns drive obesity and glucose dysfunction

Aarun Hendrickson[1], Jaden Le[1], Cristiana Meuret[1], Asmit Kumar[1], Caeley Bryan[2], Micah Glaz[2], Kendra Francis[2], David Tovar[3], Ramon Sun[4], Carlos Campos[2], Joshua Thaler[2], Michael W. Schwartz[2], Jarrad M. Scarlett[2,5], Kimberly M. Alonge[1]

[1]*Department of Medicinal Chemistry, University of Washington, Seattle, WA,* [2]*Department of Medicine, University of Washington, Seattle, WA,* [3]*Department of Psychology, Vanderbilt University, Nashville, TN,* [4]*Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,* [5]*Department of Pediatric Gastroenterology and Hepatology, Seattle Children's Hospital, Seattle, WA,*

The mediobasal hypothalamus (MBH) is a key brain region for the regulation of feeding behaviors and glucose metabolism. We previously showed that hypothalamic perineuronal nets (PNNs) are extracellular matrix structures that enmesh neurons involved in glucose and energy homeostasis. These unique PNNs are comprised of chondroitin and dermatan sulfate-glycosaminoglycans (CS/DS-GAGs) that contain differentially sulfated CS/DS isomers to create a PNN glycan "sulfation code" surrounding glucoregulatory circuits in the MBH. Each CS/DS isomer uniquely impacts the function of PNNs, thus influencing the activity of the underlying neurocircuitry. Specifically, the 6S-CS isomer has been linked to PNN destabilization and altered neurocircuit activity. Our Preliminary Data shows that in humans (n=11, ages: 22–84 yr, 3F/8M), MBH 6S-CS significantly increases with age ($p=0.035$, $R2=0.41$), suggesting that aging in the hypothalamus associates with destabilization of PNNs and altered hypothalamic neurocircuit function.

Since hypothalamic aging also associates with metabolic impairments, we wanted to determine the functional impact of increased hypothalamic PNN 6S-CS on the central control of metabolism. We performed bilateral injections of adeno-associated virus (AAV1) to overexpress 6S-sulfotransferase (AAV1-CBA-mChst3-EGFP) in GABAergic neurons within the MBH of vgat-Cre mice (n=6 mChst3 (2F/4M) and n=5 EGFP controls (2F/3M)). While EGFP control mice showed minimal changes in body weight and food intake 30 days post-injection, hypothalamic PNN 6S-CS overexpression increased % body weight (mChst3: 43.5±9.0%, EGFP: 2.2±1.3% ($p<0.0001$)) and chow intake (mChst3: 192.5±8.1g, EGFP: 147.3±6.0g ($p<0.0001$)) starting 13–15 days post-injection. Changes in % body fat were consistent with these findings: magnetic resonance imaging (MRI) before and after AAV1 administration showed 1) no difference in % body fat before surgery (mChst3: 9.6%, EGFP: 9.7% ($p=0.96$)), but 2) significant increase in % body fat gain in the hypothalamic PNN 6S-CS overexpressing mice 4 wks after viral delivery (mChst3: 28.6% (before vs 4wk, $p<0.0001$), EGFP: 11.5% (before vs 4 wk, $p=0.50$) (mChst3 vs EGFP at 4 wk, $p<0.0001$)). We also observed changes in blood glucose (BG) in the 6S-CS overexpressing mice, which showed 1) an average 20.6±4.3 mg/dL elevation of baseline BG from d14-d30 (mChst3: 155.5 mg/dL, EGFP: 134.9 mg/dL ($p=0.001$)), and 2) a glucose tolerance test (GTT) at 4 wks post-surgery showed the presence of glucose intolerance in the hypothalamic PNN 6S-CS mice (mChst3: 23627_{AUC}, EGFP: 21345_{AUC} ($p=0.07$)), compared to controls. These results provide evidence that hypothalamic PNN expression of the 6S-CS isomer is an active contributor to the onset of hyperphagia, obesity, and glucose intolerance in mice, an effect that we predict translates to the aging hypothalamus and age-associated metabolic dysfunction in humans.

(P091) Investigating elongating factor of O-Mannose glycans

Tatsuro Ikeda[1], Yoichiro Harada[2], Mariko Taniguchi-Ikeda[1]

[1]*Department of Clinical Genetics, Fujita Health University, Japan,* [2]*Department of Glyco-Oncology and Medical Biochemistry, Osaka International Cancer Institute, Japan,*

Background: Fukuyama congenital muscular dystrophy (FCMD) is a severe, intractable genetic disease that affects the skeletal muscle, eyes, and brain and is attributed to a defect in alpha dystroglycan (αDG) O-mannosyl glycosylation. Previous report showed that the basic polycyclic compound Mannan-007 (Mn007) restored glycosylation in patients cell line as well as wildtype HEK-293 cell. However, the mechanism has remained unknown. Aim: To investigate the mechanism of this change, we tested

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whether these elongated glycans are large dependent. We also tested the turnover of O-mannosyl glycans of αDG using Mn007, Result: HPLC showed that Mn007 elongated glycosylation with Large overexpression. We also found that αDG glycans were cancelled immediately by the removal of Mn007. These facts show that Mn007 might act as an inhibitor for enzymatic degradation of glycosylation of αDG glycans.

(P092) Sialylation mediates glia-neuron communication in Drosophila

Hilary Scott[1], Boris Novikov[1], Berrak Ugur[2], Brooke Allen[1], Ilya Mertsalov[1], Pedro Monagas-Valentin[1], Melissa Koff[1], Sarah Baas Robinson[3], Kazuhiro Aoki[3], Raisa Veizaj[4], Dirk Lefeber[4], Michael Tiemeyer[3], Hugo Bellen[2], Vladislav Panin[1]

[1]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, [2]Departments of Molecular and Human Genetics and Neuroscience, Baylor College of Medicine, and Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX, [3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA, [4]Department of Laboratory Medicine, Translational Metabolic Laboratory, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

Sialylation plays vital roles in numerous developmental and physiological processes in animals. The last two step of the sialylation pathway are mediated by CMP-sialic acid synthetase (CSAS) and sialyltransferase, the evolutionarily conserved enzymes that produce CMP-Sia, a sugar-nucleotide donor, and transfer sialic acid from CMP-Sia to glycan termini, respectively. While twenty different sialyltransferases work in a mammalian organism, *Drosophila* sialylation relies on a single counterpart, DSiaT. Similar to mammals, *Drosophila* has only one CSAS enzyme. In our previous studies we found that the expression of DSiaT and CSAS is restricted to the nervous system. Our experiments demonstrated that mutations in the sialylation pathway genes result in pronounced neurological phenotypes, including abnormal locomotion, heat-induced paralysis, and impaired neural excitability. Detailed analysis of DSiaT and CSAS functions revealed that these genes are expressed and functionally required in different cells, neurons and glia, respectively. The bipartite arrangement of the sialylation pathway allows for regulation of neural functions by a glia-mediated control of neural sialylation. This regulation was found to be required for heat and oxidative stress tolerance and for maintaining the normal levels of voltage-gated sodium channels. In summary, our results uncovered a unique function of the sialylation pathway in facilitating glia-neuron communication, thereby regulating neural excitability and stress tolerance. This project was supported in part by grants from NIH (NS099409 & NS075534 to V.P., and GM103490 to M.T.), TAMU CONACYT 2012-037S to V.P., and Radboud Consortium for Glycoscience to D.L.

(P093) Quality control of NOTCH and CRB2 proteins containing epidermal growth factor-like EGF repeats by xylosyl elongation of *O*-glucose glycans

Hideyuki Takeuchi[1], Yukihiro Mita[2], Yohei Tsukamoto[2], Wataru Saiki[2], Koki Kato[1], Yusuke Urata[2], Yuuki Kurebayashi[1], Akira Minami[1], Tadanobu Takahashi[1], Hideharu Hibi[2], Tetsuya Okajima[2]

[1]University of Shizuoka School of Pharmaceutical Sciences, [2]Nagoya University School of Medicine,

O-Glucose (Glc) glycosylation is an evolutionarily well-conserved form of post-translational modification: *O*-Glc monosaccharide is transferred from the sugar nucleotide UDP-Glc to a serine residue with the consensus sequence, C^1-X-S-X-P/A-C^2, in an epidermal growth factor-like (EGF) repeat by protein *O*-glucosyltransferase (POGLUT1/Rumi). In mammals, there are two enzymes, GXYLT1 and GXYLT2, that transfer xylose to this *O*-Glc monosaccharide, and one enzyme, XXYLT1, that transfers the second xylose. Previous studies have shown that *O*-Glc glycosylation is essential for the activation of Notch signaling in *Drosophila* and mice and that its abnormalities cause pathological conditions such as muscular dystrophy in humans. However, how *O*-Glc glycosylation regulates substrate protein function is not understood. Intriguingly, *Poglut1* knockout mice show not only abnormal Notch signaling but also embryonic developmental defects due to loss of Crumbs2 (CRB2) function, leading to embryonic lethality. CRB2 is a type I transmembrane protein that, like Notch, has multiple EGF repeats in the extracellular domain and regulates processes such as epithelial cell polarity formation. We comprehensively analyzed the extent of *O*-Glc glycosylation in EGF repeats of Notch and CRB2 by mass spectrometry. The extracellular domains of NOTCH1/2 and CRB2 proteins were expressed and purified in the cultured cell line HEK293T cells, and the *O*-Glc glycosylation was analyzed by mass spectrometry. Most of the EGF repeats with the consensus sequence were modified with *O*-Glc glycans. The differences in the glycan elongation of each EGF repeat indicated that xylosyl elongation is EGF-specific. In *GXYLT1* KO cells, the xylosyl elongation of several EGF repeats was reduced or lost, which was not observed in *GXYLT2* KO cells. Furthermore, we examined the role of the addition of *O*-Glc monosaccharides and subsequent xylosyl elongation of these proteins in terms of quality control of the secretory pathway, possibly the lumen of the endoplasmic reticulum. Secretion assays were performed using wild-type, *GXYLT1*, and *GXYLT2* KO HEK293T cells. Secretion of the extracellular domains of NOTCH1/2 and CRB2 proteins was not significantly altered in *GXYLT2* KO cells, but was decreased in *GXYLT1* KO cells compared to that in wild-type cells. The decrease in secretion and xylosyl elongation was rescued by co-expression of wild-type GXYLT1, but not by co-expression of GXYLT1 with a mutation in the putative catalytic domain, DXD motif. These results indicate that the contribution of GXYLT1

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is higher than that of GXYLT2 and that its xylosyltransferase activity is required for the function of GXYLT1. In summary, these results suggested that GXYLT1 predominantly regulates xylosyl elongation of *O*-Glc glycosylation of NOTCH and CRB2 in HEK293T cells, thereby controlling the trafficking of NOTCH and CRB2 to the cell surface.

(P095) Sialic Acids Specificity of Plasmodium Parasites

Patrycja Burzyńska[1], Julian Rayner[2], Ewa Jaśkiewicz[1]

[1]*Laboratory of Glycobiology, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wroclaw, Poland,* [2]*Cambridge Institute for Medical Research, University of Cambridge, Keith Peters Building, Biomedical Campus, Hills Rd, Cambridge CB2 0XY, United Kingdom,*

Plasmodium parasites which infect humans and great apes are largely host-specific but the factors that control that specificity are imperfectly understood. The goal of this work was to compare the molecular preferences of *Plasmodium* parasite ligands involved in erythrocyte invasion to sialic acids, which differ between humans and apes.

Erythrocyte recognition is largely dependent on *Plasmodium* ligands from two multigene families: erythrocyte-binding ligands (EBL) and reticulocyte-binding ligands (RBL). *P. falciparum* has four members of EBL family, including erythrocyte binding antigen 140 (EBA-140), which enable the merozoite to interact with independent human RBCs receptors. The EBA-140 merozoite ligand was shown to bind glycophorin C (GPC) a minor erythrocyte sialoglycoprotein, in a manner that is dependent on sialic acids.

The most common mammalian sialic acids (SA) are N-glycolylneuraminic acid (Neu5Gc) and N-acetytneuraminic acid (Neu5Ac), which is the metabolic precursor of Neu5Gc. Humans cannot produce Neu5Gc because of a mutation in the hydroxylase (CMAH) gene. Thus, human erythrocytes express only Neu5Ac, while most other primate erythrocytes carry a mixture of both SA, with Neu5Gc being dominant. Ape-specific *Plasmodium* parasites encode homologs of the EBLs, including EBA-140. It has been proposed that a switch in specificity from Neu5Gc to Neu5Ac might be one of the evolutionary changes which allowed ape parasites to adapt to infecting humans.

We aimed to compare the SA binding specificity of three recombinant *Plasmodium* EBA-140 ligands, from *P. falciparum* (human-infective), *P. praefalciparum and P. reichenowi* (both ape-infective) towards NeuAc and NeuGc sialic acids. We have shown that there is a clear preference for the Neu5Gc binding in all three studied species, including *P. falciparum* which largely infects humans, althoguh all ligands can also bind Neu5Ac. This suggests that at least in the case of EBA1-40, the ancestors of *P. falciparum* parasites did not need to acquire a new ability to bind Neu5Ac in order to infect humans. Thus, this study may help to illuminate the evolutionary changes in *Plasmodium* host specificity that were required and not required to allow ape parasite adapt to humans, and ultimately allowed *P. falciparum* to become the most deadly human parasite.

(P096) Leveraging Glycan-Lectin Interactions to Develop Effective Cancer Vaccines

Valerie Lensch[1], Adele Gabba[1], Robert Hincapie[2], Jeremiah A. Johnson[1], M.G. Finn[2], Laura L. Kiessling[1]

[1]*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA,* [2]*School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, USA,*

Limited treatment options for cancer patients continue to result in poor therapeutic outcomes. Cancer immunotherapies, which leverage a patient's own immune system to combat cancer, have shown promise as a new class of cancer treatment. Current immunotherapies often require *ex vivo* engineering of dendritic cells (DCs) or T cells against tumor antigens. The development of effective cancer vaccines that overcome the current limitations requires *in vivo* priming of the adaptive immune response. DCs are the primary initiators of anti-tumor immune responses due to their ability to activate specific T cells that mediate tumor cell killing. Despite the essential role of DCs in anti-cancer responses, effective methods for DC targeting and activation remain limited. We hypothesized that targeting and engagement of specific DC lectins would serve an effective strategy to activate DCs *in vivo*. The native role of DC lectins is to recognize and bind unique glycan displays on the surface of pathogens, facilitating highly efficient internalization of antigens and subsequent modulation of the immune response. To improve upon naturally occurring carbohydrate recognition motifs, we designed a synthetic mannoside ligand for the endocytic, immunostimulatory lectin DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin). Presentation of the mannose ligand on virus-like particles (VLPs) yielded a glycosylated VLP vaccine platform that bound the target lectin, elicited DC maturation, and induced proinflammatory cytokine secretion. Mice immunized with the glycosylated VLPs generated increased percentages of tumor antigen-specific T cells, leading to superior tumor growth inhibition and prolonged survival rates as compared to mice immunized with non-targeted VLPs in a mouse melanoma model. Our data demonstrate that DC lectin engagement by synthetic glycoligands can elicit robust anti-tumor responses by altering DC signaling. The development of a DC-targeting platform via lectin engagement offers an exciting framework for the advancement of cancer immunotherapies and development of new vaccines.

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(P097) Aberrant glycosylation of influenza virus: models and implications for host immune responses

Irina Alymova[1], John Cipollo[2], Wen-Pin Tzeng[1], John Barr[3], Dongxia Wang[3], Betlehem Mekonnen[3], Shane Gansebom[1,4], Ian York[1]

[1]Immunology and Pathogenesis Branch, Influenza Division, National Center for Immunization & Respiratory Diseases, Centers for Disease Control & Prevention, Atlanta, Georgia, USA, [2]Food and Drug Administration, Center for Biologics Evaluation and Research, DBPAP, Silver Spring, Maryland, USA, [3]Clinical Chemistry Branch, Division of Laboratory Science, Centers for Disease Control & Prevention, Atlanta, Georgia, USA, [4]General Dynamics Information Technology, Atlanta, Georgia, USA

Influenza viruses cause substantial morbidity and mortality globally. The surface proteins (hemagglutinin [HA] and neuraminidase [NA]) of currently circulating seasonal influenza A viruses (IAV) are heavily glycosylated. Being an intracellular parasite, IAV uses cellular machinery for N-linked glycosylation (NLG) of the HA and NA. People with cancer and other systemic diseases often have metabolic dysregulation of cellular glycosylation, leading to reduced or increased glycosylation and/or changes in glycoforms of their proteins. These people also tend to have more severe influenza virus disease, with a poor immune response to the virus. Mass spectrometry (MS) analysis of A(H3N2)2013 IAV isolated from cells with normal glycosylation show that at least 10 of 12 NLG sites of HA and 5 of 8 sites of NA have relatively high (from 70% to 100%) glycan occupancy, while at least 3 NLG sites of either HA or NA of this IAV have low (from 0% to 25%) glycan occupancy. Aberrantly reduced cellular glycosylation can diminish glycosylation at highly occupied sites and, conversely, aberrantly enhanced cellular glycosylation can increase glycosylation at sites of HA and NA with low occupation. We investigated the consequences of aberrant cellular glycosylation for the glycome, biology, and immune responses to influenza virus. To model effects of abnormally reduced glycosylation, we treated MDCK cells infected with A(H3N2)2013 IAV with an oligosaccharyltransferase inhibitor, NGI-1. To enhance A(H3N2)2013 IAV glycosylation and change glycoform usage, we passaged the virus through A549 human lung carcinoma cells. Our MS data show that aberrant cellular glycosylation is reflected in the glycosylation pattern of influenza virus isolated from those cells. There were no sequence changes observed in the N-linked sequons of glycome-modified virus. As evidenced by IAV with abnormally reduced glycosylation, glycome-modified virus can be replicatively competent and able to efficiently infect new cells. Both innate and adaptive immune responses toward glycome-modified IAV can be reduced. The respiratory tract innate immune collectin SP-D required higher concentrations to neutralize glycome-modified virus than for virus with normal glycosylation, and glycome-altered virus also generated lower total and protective antibody responses in mice than did IAV with normal glycosylation. Reduced immune responses of the host toward glycome-altered virus might be one reason for the increased viral load in the respiratory tract of people with metabolic disorders of glycosylation. Thus, aberrant cellular glycosylation can lead to sequence-neutral changes in the influenza virus glycome, and these glycome-modified viruses may be less well recognized by the host innate and adaptive immune system resulting in more severe influenza disease. This knowledge will serve as the foundation for future studies to interrogate the role of aberrant virus glycosylation in the influenza disease pathogenesis.

(P098) Inhibition on the Interactions between Viral Protein and Glycosaminoglycan by Sulfated Glycans

Fuming Zhang[1], Peng He[1], Yuefan Song[1], Deling Shi[1], Weihua Jin[1], Ke Xia[1], Vitor H. Pomin[2], Robert J. Linhardt[1], Jonathan S. Dordick[1]

[1]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, United States, [2]Department of BioMolecular Sciences, Research Institute of Pharmaceutical Sciences, The University of Mississippi, Oxford, MS, United States,

Many viruses use the heparan sulfate (HS) on the surface of host cells as co-receptors for attachment and initiating cell entry. Therefore, virion-HS interactions have been targeted for developing broad-spectrum antiviral therapeutics. Here we report the potential anti-SARS-CoV-2 and monkeypox virus (MPXV) activities of sulfated glycans including Pentosan Polysulfate (PPS) and Mucopolysaccharide Polysulfate (MPS), and eight defined marine sulfated glycans (three fucosylated chondroitin sulfates and three sulfated fucans extracted from the sea cucumber species and the sea urchin, and two chemically desulfated derivatives). The inhibition of these sulfated glycans on interactions between the receptor-binding domain (RBD) of S-protein of SARS-CoV-2 (both WT and different variants) and heparin, interactions between MPXV A29 and A35 protein and heparin were evaluated using surface plasmon resonance (SPR). The SPR results demonstrate sulfated glycans show strong inhibition on these interactions. The study of molecular interactions between viral proteins and host cell GAGs is important in developing therapeutics for the prevention and treatment of COVID-19 and monkeypox.

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(P099) An in-depth multi-dimensional analysis of recombinant CD52 glycopeptide

Nicholas DeBono[1,2,3], Muhammad A. Zenaidee[4], Esther Bandala-Sanchez[5], Edward S.X. Moh[1,3], Leonard C. Harrison[5], Nicolle H. Packer[1,3,4]

[1]*School of Natural Sciences, Macquarie University, Sydney, NSW, Australia,* [2]*CSIRO Synthetic Biology Future Science Platform, Canberra, ACT, Australia,* [3]*ARC Centre of Excellence in Synthetic Biology, Macquarie University, Sydney, NSW, Australia,* [4]*Australian Proteome Analysis Facility, Macquarie University, Sydney, NSW, Australia,* [5]*The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia,*

Soluble CD52 is an immune regulator which initially sequesters pro-inflammatory high mobility group box protein 1 (HMGB1) and inhibits immune responses (1). Recombinant CD52-Fc has been shown to act as a broad anti-inflammatory agent, dampening both adaptive (1) and innate (2) immune responses. Human soluble CD52 is a short glycopeptide comprising 12 amino acids (GQNDTSQTSSPS) carrying an N-linked sialylated complex glycan at Asn3 and with potential O-linked glycosylation sites on serine/threonine residues. Previously, we demonstrated that specific glycosylation of CD52 is essential for its immunosuppressive function (1), with terminal α-2,3-linked sialic acids playing a key role in binding to the inhibitory SIGLEC-10 receptor leading to T-cell suppression (1, 2). However, production of recombinant proteins in different host cell lines for therapeutic use will result in expression of different glycoforms compared to the native protein, which can reduce its biological activity. Using top-down and traditional high-resolution mass spectrometry, we were able to confidently identify large numbers of glycoforms on recombinant CD52 produced in HEK293 cells. Combining this glycopeptide analysis with information on PNGase F released N-glycans using porous graphitised carbon LC-MS/MS in negative ion mode, we characterised the glycan structures comprising the various glycoforms on both native and recombinant CD52. Additionally, analysis of the glycopeptide after release of the N-glycans allowed the glycosylation site and monosaccharide composition of the O-glycosylation to be determined. As glycosylation of CD52 is essential to its function, accurate identification of its glycoforms will inform the development of CD52 as a therapeutic agent.

(P100) Digestion of *N*-glycolylneuraminic acid-containing glycans in the diet by sialidase

Hatsune Nakao[1], Akira Minami[1], Ayumi Matsumoto[1], Yoshio Hirabayashi[2], Tadamune Otsubo[3], Kiyoshi Ikeda[3], Yuuki Kurebayashi[1], Tadanobu Takahashi[1], Hideyuki Takeuchi[1]

[1]*Department of Biochemistry, Graduate School of Pharmaceutical Sciences, University of Shizuoka,* [2]*RIKE,* [3]*Department of Organic Chemistry, School of Pharmaceutical Sciences, Hiroshima International University,*

The major molecular species of sialic acid present in nature are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Humans are unable to biosynthesize Neu5Gc due to a gene deletion of the human cytidine monophospho-*N*-acetylneuraminic acid hydroxylase. However, it has been reported that Neu5Gc can be detected in some human vascular endothelial cells and epithelial carcinomas. Anti-Neu5Gc antibodies produced by exposure to Neu5Gc elicit chronic inflammation, atherosclerosis, and carcinomas. It is assumed that Neu5Gc derived from red meat and other dietary sources is absorbed by the human body. To understand the detailed mechanism by which Neu5Gc is absorbed into the human body, we investigated the digestion of Neu5Gc-containing glycans in the diet by focusing on the function of sialidase.

Sialidase cleaves sialic acid residues from glycans such as glycoproteins and glycolipids. First, we examined the release of sialic acid from glycans during digestion by analyzing the enzymatic activity of sialidase. Sialidase activity was detected in human saliva and in the stomach, small intestine, and large intestine of rats. Sialic acid is chemically hydrolyzed under acidic conditions to release it from the glycan. Thus, we also examined the effect of gastric acid on the digestion of Neu5Gc-containing glycans in the diet. Since beef is rich in Neu5Gc, hamburger steaks made from ground beef were homogenized and then soaked in a hydrochloric acid solution (pH 1.2) that mimics gastric acid for 4 hours. Around 1 hour after the start of acid treatment, Neu5Gc started to be released from the glycans, and after 4 hours, Neu5Gc was released from 30% of the Neu5Gc-containing sugar chains. In the intestinal epithelial barrier model constructed with the human intestinal epithelial cell line, free Neu5Gc added to the intestinal lumen side permeated to the lamina propria side. Next, we analyzed changes in the concentration of Neu5Gc in blood when hamburger steaks were consumed by humans after fasting. Neu5Gc levels in the blood reached a maximum of 60 minutes after ingestion and returned to pre-ingestion levels after 240 minutes. These results suggest that dietary Neu5Gc is cleaved from glycans in the diet by sialidase in saliva, stomach, small and large intestine, and by gastric acid, and absorbed into the blood in the intestinal tract.

(P101) Structure and Function of a Novel Family of IgG-specific Endoglycosidases

Diego E. Sastre[1], Eric J. Sundberg[1]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, USA,*

Endo-β-*N*-acetylglucosaminidases, or endoglycosidases, produced by a wide range of organisms catalyze the hydrolysis of *N*-linked glycans on glycoproteins. The vast majority of endoglycosidases recognize their glycoprotein substrates by glycan-specific,

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but protein-nonspecific, mechanisms. However, a small subset of endoglycosidases specifically hydrolyze the Asn297-linked glycan on IgG antibodies. This glycan is the major molecular determinant of Fc γ receptor and complement C1q binding by IgG antibodies, interactions that in turn trigger antibody-mediated effector functions that are critical for the signaling properties of these antibodies. Accordingly, IgG-specific endoglycosidases could be useful for the treatment or prevention of diseases or conditions mediated by IgG antibodies including, but not limited to, autoimmunity and transplantation rejection. The most well-characterized IgG-specific endoglycosidases are multi-domain proteins belonging to a family of enzymes exemplified by EndoS and EndoS2, which are secreted by various strains of *Streptococcus pyogenes* as an immune evasion strategy. Using sequence similarity network (SSN) analysis and protein folding algorithms, we identified a new family of single-domain endoglycosidases secreted by *Corynebacterium* species that we showed to be IgG-specific using intact glycoprotein liquid chromatography-mass spectrometry (LC-MS) techniques. We combined X-ray crystallography, site-directed mutagenesis, molecular interaction and LC-MS methods to define the molecular mechanism by which a prototypical member of this novel IgG-specific endoglycosidase family hydrolyzes *N*-linked glycans strictly from IgG antibodies. Furthermore, we conducted *in vivo* assays employing humanized mouse models that demonstrated the remarkable efficacy of this prototypical single-domain IgG-specific endoglycosidase in mitigating pathologies that rely on IgG-mediated effector functions.

(P102) The mucin-type glycosyltransferase Toxoplasma gondii GalNAc-T3 uses a distinct mechanism from its host homologues to O-glycosylate bradyzoite cyst wall proteins

Pranav Kumar[1], Tadakimi Tomita[2], Thomas Gerken[3], Yong Sok Lee[4], Louis Weiss[2,5], Nadine Samara[1]

[1]*Structural Biochemistry Unit, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, 20892, USA,* [2]*Departments of Pathology, Albert Einstein College of Medicine, Bronx, 1300 Morris Park Avenue, New York, 10461, USA,* [3]*Departments of Biochemistry and Chemistry, Case Western Reserve University, Cleveland, OH, 44106, USA,* [4]*Bioinformatics and Computational Biosciences Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland 20892, USA,* [5]*Departments of Medicine, Albert Einstein College of Medicine, Bronx 1300 Morris Park Avenue, New York, 10461, USA,*

Mucin-type O-glycosylation results in the addition of O-glycans to Thr/Ser residues on proteins and is conserved across eukaryotes, including Toxoplasma gondii (T. gondii), an obligate intracellular protozoan parasite that infects one-third of the world's population and causes toxoplasmosis. Host infection by T. gondii is associated with the formation of latent cysts in the central nervous system that are enveloped with a structure called the cyst wall, whose rigidity is imparted by the O-glycosylation of the mucin domain of the cyst wall protein CST1. Reduced sequence homology between T. gondii and metazoan GalNAc-Ts hints that an evolutionary divergence may have occurred in substrate recognition and enzyme function. X-ray crystal structures of T. gondii-GalNAc-T3 in complex with glycopeptides from 2.5–2.9 Å resolution show that the substrate specificity of T. gondii-GalNAc-T3 is controlled by a unique GalNAc binding pocket that recognizes an existing GalNAc on a substrate one amino acid C-terminal to the acceptor site. The structures also reveal a 2nd novel metal binding site that is coupled to the GalNAc binding pocket. Mutations of residues involved in both GalNAc and 2nd metal binding reduce enzymatic activity in vitro and disrupt cyst wall formation in cellulo. Our studies illustrate how GalNAc-T evolution from protozoan pathogen to mammalian host lays the framework for specifically inhibiting T. gondii-GalNAc-Ts in toxoplasmosis.

(P103) Deciphering the Multifaceted Roles of Glycans in Viral Glycoproteins Using the Computational Microscope

Lorenzo Casalino[1], Rommie E. Amaro[1]

[1]*Department of Molecular Biology, University of California San Diego, La Jolla, CA, United States,*

Glycans play pivotal roles in diverse biological processes, including viral infections. Glycosylation profoundly influences viral glycoprotein structure, function, and immune recognition, thus shaping the course of viral pathogenesis. The intrinsic flexibility of glycans often limits their full three-dimensional structural characterization via imaging techniques such as cryo-electron microscopy or X-ray crystallography. This inherent challenge underscores the importance of employing computational approaches to provide a unique lens to study the dynamic behavior of glycans and their role in viral glycoproteins, enabling the exploration of properties that may remain cryptic to experimental approaches alone. Here, by employing cutting-edge in-silico modeling and all-atom molecular dynamics, we delve into the realm of viral glycoproteins, including SARS-CoV-2 spike, influenza hemagglutinin (HA) and neuraminidase (NA), HIV-1 Env, and Lassa virus glycoprotein complex (GPC), to present an atomic-level understanding of their glycan shields and dissect their many roles in viral pathogenesis. By probing microsecond timescales, we unveil vulnerabilities in the glycan shield across different class I fusion glycoproteins. Examining the SARS-CoV-2 spike glycoprotein, we discovered, beyond shielding, the functional role of specific N-linked glycans (N165, N234, N343) in priming the spike for host cell infection. Expanding our focus on other viruses, we explore the pivotal contribution of glycans in shaping the dynamics, functionality, and immunogenicity of influenza HA and NA, HIV-1 Env, and Lassa virus GPC, providing an atomic-level perspective of glycans as essential determinants for understanding and addressing viral diseases. Overall, our

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simulations significantly advanced the knowledge of viral glycoproteins' glycan shield and its many roles, opening new avenues for vaccine design and drug development.

(P104) TSG6 hyaluronan matrix remodeling dampens the inflammatory response during colitis

Nansy Albtoush[1,4], Kimberly A. Queisser[1,4], Ash Zawerton[4], Mark E. Lauer[5,*,†], Ellen J. Beswick[6], Aaron C. Petrey[1,2,3,4]

[1]University of Utah Molecular Medicine Program, Salt Lake City, Utah, 84112, [2]Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah, 84132, [3]Division of Gastroenterology, Department of Internal Medicine, University of Utah, Salt Lake City, Utah, USA, [4]Lerner Research Institute, Department of Inflammation & Immunity, Cleveland Clinic, Cleveland, OH 44195, USA, [5]Lerner Research Institute, Department of Biomedical Engineering, Cleveland Clinic, Cleveland, OH 44195, USA, [6]Division of Gastroenterology, Department of Internal Medicine, University of Kentucky, Lexington, KY, United States,

[†]Deceased

In response to tissue injury, alterations of the extracellular matrix (ECM) can directly influence the inflammatory response and contribute to disease progression or resolution. During inflammation, the glycosaminoglycan hyaluronan (HA) can become modified by the enzymatic activity of tumor necrosis factor stimulated gene-6 (TSG6). TSG6 covalently transfers heavy chain (HC) proteins from inter-α-trypsin inhibitor (IαI) to HA in a transesterification reaction and to date is the only known HC:HA transferase. By modifying the HA matrix, TSG6 generates HC:HA complexes that are implicated in mediating both protective and pathological responses. Inflammatory bowel disease (IBD) is a lifelong chronic disorder with well-described remodeling of the ECM and increased mononuclear leukocyte influx into the intestinal mucosa. Deposition of HC:HA matrices is an early event in inflamed gut tissue that precedes and promotes leukocyte infiltration. However, the mechanisms by which TSG6 contributes to intestinal inflammation are not well understood. The aim of our study was to understand how the TSG6 and its enzymatic activity contributes to the inflammatory response in colitis. Our findings indicate that inflamed tissues of IBD patients show an elevated level of TSG6 and increased HC deposition and that levels of HA strongly associate with TSG6 levels in patient colon tissue specimens. Additionally, we observed that mice lacking TSG6 are more vulnerable to acute colitis and exhibit an aggravated macrophage-associated mucosal immune response characterized by elevated pro-inflammatory cytokines and chemokines and diminished anti-inflammatory mediators including IL-10. Surprisingly, along with significantly increased levels of inflammation in the absence of TSG6, tissue HA levels in mice were found to be significantly reduced and disorganized, absent of typical "HA-cable" structures. Using TSG6-activity neutralizing antibodies or small molecule inhibitors of TSG6, our data indicates that TSG6 plays an essential role in retaining inflammation-induced HA on the cell surface. Further, inhibition of TSG6 HC-transferase activity leads to a loss of cell surface HC:HA and leukocyte adhesion, indicating that the enzymatic functions of TSG6 are a major contributor to stability of the HA ECM during inflammation. Finally, using biochemically generated HC:HA matrices derived by TSG6, we show that HC:HA complexes can attenuate the inflammatory response of activated monocytes. In conclusion, our data suggests that TSG6 dependent modification of HA exerts a tissue-protective, anti-inflammatory effect via the generation of HC:HA complexes.

(P105) Aberrant O-glycosylation of IgA1 and complement in IgA nephropathy: Glomerular immunodeposits of patients with histologically active disease exhibit elevated colocalization of IgA and complement C3

Lea Novak[1], Stacy D. Hall[1], Graham L. Gurganus[1], Dana V. Rizk[1], Bruce A. Julian[1], Mark Haas[2], Jan Novak[1]

[1]Departments of Microbiology and Medicine, University of Alabama at Birmingham, Birmingham, AL, USA, [2]Department of Pathology, Cedars-Sinai Medical Center, Los Angeles, CA, USA,

IgA nephropathy (IgAN), a common primary glomerulonephritis, often leads to kidney failure. Diagnostic routine immunofluorescence microscopy reveals glomerular immunodeposits containing IgA and usually complement C3 (C3), with variable presence of IgG and/or IgM. We have postulated that glomerular immunodeposits in IgAN originate from the IgA1-containing circulating immune complexes (CIC). IgA1 in these CIC is aberrantly glycosylated; some hinge-region O-glycans are galactose-deficient (Gd-IgA1). This Gd-IgA1 is recognized by IgG autoantibodies to form immune complexes. Additional proteins, such as C3, can be subsequently attached. CIC deposited in the glomeruli induce kidney injury. This hypothesis is supported by the findings that glomerular immunodeposits in IgAN are enriched for Gd-IgA1 glycoforms and IgG autoantibodies specific for Gd-IgA1. In this study, we used high-resolution confocal microscopy (HRCM) to evaluate colocalization of C3, IgA, and IgG in glomerular immunodeposits of patients with IgAN and correlated results with histologic features of kidney injury graded according to the Oxford MEST-C scores.

Frozen-tissue sections of remnant kidney-biopsy specimens from 17 IgAN patients were stained using fluorochrome-labeled reagents specific for human IgA, IgG, and C3. Using HRCM, z-stack images of glomeruli were acquired (objective 60x). For each slide, 60 regions of interest (ROIs) were selected in multiple optical planes in areas with glomerular C3 staining. Pearson's

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correlation coefficient (PCC) colocalization data were collected for each C3-IgA, IgA-IgG, and C3-IgG pairwise comparison and mean values were calculated. Tissue injury was assessed using the Oxford classification scoring: mesangial (M) and endocapillary (E) hypercellularity, segmental glomerulosclerosis (S), interstitial fibrosis/tubular atrophy (T) and presence of crescents (C). To correlate the colocalization data with Oxford scores, patients were divided in paired groups: M0/M1, E0/E1, S0/S1, T0 /T1+T2, and C0/C1+C2. Differences in mean PCC in C3-IgA, IgA-IgG, and C3-IgG paired groups were statistically evaluated. In addition, proteins from the immunodeposits of 3 other remnant IgAN kidney-biopsy specimens were extracted and analyzed by SDS-PAGE immunoblotting with C3-specific antibody.

IgA, IgG, and C3 were detected in glomerular immunodeposits of all 17 IgAN patients by HRCM. Colocalization of C3-IgA was higher in IgAN patients with Oxford scores M1 ($p=0.036$), E1 ($p=0.025$), and C1+C2 ($p=0.018$) compared to M0, E0, and C0 scores, respectively. C3-IgA colocalization for S0 vs. S1 and T0 vs. T1+T2 did not differ. IgA-IgG and IgG-C3 colocalizations did not differ when correlated with Oxford scores. Immunoblotting confirmed the presence of C3 in extracts from the immunodeposits.

In conclusion, elevated C3-IgA colocalization in glomerular immunodeposits was associated with more severe disease, implicating complement in the kidney injury in IgAN.

(P106) Protein *O*-GlcNAcylation regulates the homeostasis of innate B lymphocytes

Yi-Hsuan Chang[1], Kuo-I Lin[2], Takashi Angata[1,3]

[1]Institute of Biochemical Sciences, National Taiwan University, [2]Genomics Research Center, Academia Sinica, [3]Institute of Biological Chemistry, Academia Sinica,

Protein O-GlcNAcylation is a type of intracellular glycosylation, which is synthesized by O-GlcNAc transferase (OGT) that transfers the GlcNAc moiety from UDP-GlcNAc to serine/threonine residues of cytoplasmic and nuclear proteins. O-GlcNAcylation is vital for the development, proliferation, differentiation, and functions of immune cells. We previously showed that the survival of mature B cells in the bone marrow as well as the activation of follicular B cells in the spleen depend on O-GlcNAcylation of Lyn, which is critically involved in the B-cell activating factor (BAFF) and B cell receptor (BCR) signaling pathways. A subtype of B cells called B-1 cells develop from progenitors in the fetal liver, and the majority of them migrate and thereafter reside in the body cavity in adulthood. When they encounter pathogen-associated molecular patterns (PAMPs), B-1 cells are activated and egress to the spleen to generate high levels of natural antibodies and cytokines required for timely defense against pathogens. Given that B-1 cells are not responsive to BCR ligation despite high levels of surface IgM expression, and that they do not require BAFF signaling for survival, we here study whether B-1 cells depend on protein O-GlcNAcylation for survival, proliferation, and functions. Intracellular O-GlcNAc staining revealed that splenic B-1 cells have higher O-GlcNAcylation levels than peritoneal B-1 cells in WT mice. Using B cell-specific *Ogt* knockout (B-KO) mice, we found that the loss of *Ogt* significantly reduced the B-1 cell population in the body cavity. However, OGT deficiency did not impair the survival of B-1 cells. Furthermore, the BrdU incorporation rate was not reduced in B-1 cells from B-KO mice, demonstrating that B-1 cell duplication was not impaired by OGT deficiency. Splenic B-1 cell transcriptome analysis further revealed genes involved in cell migration and adhesion are regulated by O-GlcNAcylation. Specifically, the expression of several integrins was decreased on the B-1 cell surface when *Ogt* was deleted, suggesting the role of O-GlcNAcylation in helping B-1 cell migration. Indeed, results from the transwell assay demonstrated that the loss of O-GlcNAcylation impaired B-1 cell migratory ability. Taken together, we here found a role for O-GlcNAcylation in maintaining B-1 cell homeostasis via promoting the migration of B-1 cells into the body cavity. Further study will focus on the search for O-GlcNAcylated proteins involved in the regulation of surface integrin expression and trafficking to elucidate how O-GlcNAcylation affects B-1 cell migration.

(P107) Discovery of sulfatide as a glycosphingolipid with specific binding properties to norovirus

Bunta Tsukamoto[1], Yuuki Kurebayashi[1], Yusuke Abe[1], Yoshiki Wakabayashi[1], Akira Minami[1], Tadanobu Takahashi[1], Takashi Suzuki[1], Hideyuki Takeuchi[1]

[1]Department of Biochemistry, Graduate School of Pharmaceutical Sciences, University of Shizuoka,

Norovirus is the leading causative pathogen of infectious gastroenteritis, causing more than 700 million infections and 200,000 deaths yearly. To date, there is still no effective drug or vaccine. Despite serious health concerns, little is known about the norovirus infection cycle, including cell binding and invasion. Therefore, in this study, we aimed to search for host factors that bind to norovirus to elucidate the molecular mechanisms of norovirus infection. Previous studies by several groups have shown that gastroenteritis viruses, such as rotaviruses and adenoviruses, utilize glycosphingolipids as attachment factors and entry receptors. Therefore, we focused on glycosphingolipids as binding receptors of norovirus, searching for glycosphingolipids that bind to norovirus.

First, we explored glycosphingolipids that bind to norovirus strain GV.CW1 with ELISA. GW.CW1 norovirus was cultured in RAW264.7 cells and purified from the culture media by ultrafiltration. Using microtiter plates coated with 12 glycosphingolipids,

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mainly found in human small intestinal epithelial cells, we detected norovirus which bound to these glycosphingolipids with an anti-norovirus antibody. We found that 3-*O*-sulfogalactosylceramide (sulfatide), a type of glycosphingolipid abundantly present in the body, showed high binding activity to norovirus. Next, we hypothesized that one of the norovirus proteins, Viral Protein 1 (VP1), is involved in the sulfatide binding because VP1 is a major capsid protein responsible for host-cell interaction. To test this hypothesis, we expressed a His-tagged version of VP1 from several strains GI.6, GII.4, and GV.CW1 in HEK293T cells, purified VP1 from the cell lysate by Ni-NTA affinity chromatography, and conducted the same ELISA analysis. We found that VP1 from all strains binds to sulfatide. Since VP1 comprises the P and S domains, we next investigated which domain of VP1 binds to sulfatide. We expressed the P and S domain with a His tag in HEK293T cells and *E. coli*, respectively, and purified these domains from the cell lysate by Ni-NTA affinity chromatography. The ELISA analysis with the VP1, P domain, and S domain showed that the P domain, located on the viral surface side of VP1, exhibits as high binding as VP1, suggesting that norovirus binds to sulfatide via the P domain of VP1. Moreover, we found that the P domain exhibited lower binding activity to sulfatide structural analogs than sulfatide, suggesting that norovirus specifically recognizes and binds to sulfatide structures.

In conclusion, our results indicate that sulfatide may be an attachment receptor for norovirus. We are generating cells overexpressing or knocking out sulfatide biosynthetic and metabolic enzymes. We plan to use these cells to elucidate the effect of sulfatide on norovirus infection. The results of this research will contribute to the understanding of the molecular mechanism of norovirus infection and the discovery of new drug targets.

(P108) Glycan Remodeling on the Cell Surface and Deep Mutational Scanning to Identify Fc Variants with Altered Affinity to Fc Gamma Receptor 3a

Ting Xu[1], Tatiana A. Chernova[1], Eric J. Sundberg[1]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA,*

Antibodies perform two distinct functions using two regions of the molecule – recognition by fragment antigen-binding regions (Fab) and signaling by fragment crystallizable (Fc) regions. Fc-dependent signaling mechanisms, or antibody-mediated effector functions, occur through Fc interactions with Fc receptors on immune cells. The linkage of a complex biantennary glycan to a conserved *N*-linked glycosylation site on Asn297 allows IgG antibodies to efficiently interact with Fc γ receptors (FcγRs); antibodies lacking glycosylation exhibit reduced effector functions. Correlations between IgG-FcγR binding affinities and responses to therapy highlights the importance of engineering antibodies with varying binding affinity to FcγRs.

We aim to combine screening of cell surface display library with glycan remodeling of Fc domains in order to evaluate all protein and glycan variations that influence binding to FcγRs and, subsequently, Fc-mediated effector functions. To validate the system, we first generated a stable cell line expressing IgG1 Fc on a cell surface and tested for FcγR binding followed by fluorescent staining. Cell surface-derived dissociation constants measured by our technique are consistent with published binding affinities determined by surface plasmon resonance analysis, indicating that a fully functional IgG1 Fc homodimer was displayed on the HEK293T cell surface.

Next, we sought to manipulate cell surface display by altering IgG glycosylation. Mammalian cells typically produce IgG1 antibodies with a heterogeneous mixture of core complex type *N*-glycans. However, individual IgG1 glycoforms are known to exhibit distinct FcγR binding and effector function properties. In order to produce distinct IgG1 glycoforms in our cell surface display, we employed chemoenzymatic synthesis glycan remodeling of HEK293T cell-displayed IgG1 Fc by hydrolyzing Asn297-linked glycans with the IgG-specific endoglycosidase EndoS2, followed by transglycosylation with the glycosynthase variant EndoS2$_{D184M}$ and an oxazoline derivative glycan, Ox-S2G2. As a proxy for glycan remodeling, we measured FcγR3a binding by flow cytometry before glycan remodeling, after glycan hydrolysis, and after transglycosylation and observed binding, reduced binding, and restored binding, respectively.

By combining cell surface display and glycan remodeling with deep mutational scanning libraries, we will be able to identify Fc variants for each protein and glycan variations that bind with differing affinities to FcγRs.

(P109) Core fucosylation of N-linked glycans is necessary for IL-2/15 signaling and the differentiation of functional effector memory CD8+ T cells

Mahmoud Abdelbary[1], Jake Harbour[1], Samantha Fancher[1], Jack McLean[1], Jeffrey Nolz[1]

[1]*Molecular Microbiology and Immunology, Oregon Health & Science University,*

Core fucosylation (CF) of N-linked glycans is indispensable for maintaining normal organ function and physiology of mammals, but important roles for this post-translational modification within the immune system remain largely undefined. Following viral infections, we find that expression of fucosyltransferase 8 (*Fut8*) and CF of N-linked glycans are both increased significantly by virus-specific CD8+ T cells, suggesting CF may be critical for regulating functional attributes of activated T cells. *Fut8* is the only enzyme in mammals that catalyzes CF, thus, we generated mice harboring a floxed version of the *Fut8* gene, allowing us to specifically eliminate CF only within conventional T cells. Following acute LCMV infection, antigen-specific *Fut8*$^{-/-}$ CD8^{+} T cells expanded normally, but rapidly transitioned into memory precursors (CD127^{+}KLRG1^{-}) and did not generate highly

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cytotoxic, terminally differentiated effector memory T cells (KLRG1^{+}CD127^{-}). Unexpectedly, whole genome transcriptional profiling found that lack of CF did not cause significant changes in gene expression by CD8+ T cells following activation. Rather, we discovered that *Fut8*$^{-/-}$ CD8+ T cells were unable to express the b chain (CD122) of the trimeric IL-2 receptor signaling complex, whereas expression of the a (CD25) and g_c (CD132) chains were not affected. In agreement with this observation, we find that both IL-2 and IL-15 signaling by *Fut8*$^{-/-}$ CD8+ T cells was impaired compared to WT T cells and exhibited diminished proliferation and secondary expansion following re-infection. Given that Il-2/15 signaling has been implicated in a number of immunological processes, our findings introduce core fucosylation of N-linked glycans as a novel and critical regulator of CD8+ T cell biology that is necessary for the generation of effector memory CD8+ T cells that provide protection against re-infections.

(P110) The α2,8-disialyl Motif Modulates B-cell Receptor Signaling

Ho-Yang Tsai[1,3], Po Long Hsiao[2], Wan-Jung Wu[1], Takashi Angata[2], Kuo-I Lin[1]

[1]*Genomics Research Center, Academia Sinica, Taipei, Taiwan,* [2]*Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan,* [3]*Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan,*

Glycans play various roles in B cell receptor (BCR) signaling. BCR is composed of membrane-bound immunoglobulin M (IgM) and CD79a/b heterodimer. IgM cannot induce signaling by itself upon crosslinking. Instead, the immunoreceptor tyrosine-based activation motif (ITAM) on both CD79a and CD79b contributes to BCR signaling, which can be regulated by other various surface components. CD45 is a glycan-rich receptor-type tyrosine phosphatase involved in the removal of inhibitory phosphorylation on the Src kinase family. CD22, a sialic acid-binding immunoglobulin-type lectin (Siglec) primarily expressed on B cells, recognizes glycans of CD45 on B cells. Inhibitory signaling via the immunoreceptor tyrosine-based inhibition motif (ITIM) of CD22 is spatially segregated from BCR upon interaction with CD45. Sialic acid is a terminal sugar found in the glycans attached to glycoprotein and gangliosides. Terminal sialic acids on both N- and O-glycans can modulate protein-protein interaction. The biosynthesis of the α2,8-disialyl motif is catalyzed by alpha-N-acetylneuraminide α2,8-sialyltransferase (ST8Sia) family. We previously showed that ST8Sia6 is dramatically upregulated during B cell differentiation, and the disialic acid motif is abundantly expressed on the surface of plasma cells. The physiological importance of disialic acid in B cells is not known. We generated B cell-specific ST8Sia6 knockout mice, and found that these mice display abnormal follicular B (FoB) cell activation and enhanced IgM humoral immune response to T cell-independent stimulation. On the other hand, FoB cell development and germinal center (GC) responses were unaffected. These results suggest that the α2,8-disialyl motif influences IgM-associated signaling. We further found that ST8Sia6-deficient FoB cells show reduced binding with Siglec-E, a leading candidate lectin recognizing the α2,8-disialyl motif. However, Siglec-E was not expressed on FoB cells. Thus, we suspect that the α2,8-disialyl motif may modulate the protein function independent of lectins or interact with other lectin. Thus far, we have identified several candidate proteins modified by α2,8-disialic acid. CD45 is one of them as it binds less with Siglec-E in ST8Sia6 knockout B cells. α2,8-disialyl motif deficiency did not affect IgM, CD45, or CD22 expression. Further studies will focus on the glycan profiles on CD45 and the mechanism by which the α2,8-disialyl motif deficiency affects the spatial distribution of IgM, CD45, and CD22 on the B cell surface.

(P111) Inhibiting N-glycan processing increases the antibody binding affinity and effector function of human natural killer cells

Maria Carolina Rodriguez Benavente[1], Harrison B. Hughes[1], Paul G. Kremer[1], Ganesh P. Subedi[2], Adam W. Barb[1,2,3,4,*]

[1]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA,* [2]*Roy J Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA,* [3]*Department of Chemistry, University of Georgia, Athens, GA,* [4]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA*

Novel approaches are required to improve the efficacy of immunotherapies and increase the proportion of patients who experience a benefit. Antibody-dependent cell-mediated cytotoxicity (ADCC) contributes to the efficacy of many monoclonal antibodies therapies. Natural killer (NK) cells mediate ADCC, though responses are highly variable and depend on prior treatment as well as other factors. Thus, strategies to increase NK cell activity are expected to improve multiple therapies. Both cytokine treatment and NK cell receptor engineering are being explored to increase ADCC. Post-translational modifications, including glycosylation, are widely recognized as mediators of cellular processes but minimally explored as an alternative strategy to increase ADCC. We evaluated the impact of treatment with kifunensine, an inhibitor of asparagine-linked (N-)glycan processing, on ADCC using primary and cultured human NK cells. We also probed affinity using binding assays and CD16a structure with nuclear magnetic resonance spectroscopy.Treating primary human NK cells and cultured YTS-CD16a cells with kifunensine doubled ADCC in a CD16a-dependent manner. Kifunensine treatment also increased the antibody-binding affinity of CD16a on the NK cell surface. Structural interrogation identified a single CD16a region, proximal to the N162 glycan and the antibody-binding interface, perturbed by the N-glycan composition. The observed increase in NK cell activity following kifunensine treatment synergized with afucosylated antibodies, further increasing ADCC by an additional 33%.These results demonstrate native N-glycan processing is an important factor that limits NK cell ADCC. Furthermore, optimal antibody and CD16a glycoforms are defined that provide the greatest ADCC activity.

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(P112) Glycan analysis of HIV-1 envelope glycoproteins reveals site-specific impacts on antibody recognition

Peng Zhao[1], Seoho Oh[2], Rui Kong[2], Lance Wells[1]

[1]Complex Carbohydrate Research Center, Department of Biochemistry and Molecular Biology, and Department of Chemistry, University of Georgia, Athens, Georgia, 30602, USA, [2]Department of Pathology and Laboratory Medicine, Emory Vaccine Center, Emory University, Atlanta, Georgia, 30329, USA,

The extensively N-glycosylated Envelope (Env) glycoprotein of human immunodeficiency virus type 1 (HIV-1) is an important target for the development of HIV vaccines. The multitude of N-glycosylation sites on the Env trimer serve as a glycan shield for the virus protecting it from the humoral immune responses. These surface glycans also serve as a key antigenic epitope for many of the broadly neutralizing antibodies (bNAbs). Here, we present glycosylation profiles of Env trimers purified from human 293F and CHO cell lines. Our glycoproteomic data showed that more than 70% of the sites were highly occupied (with occupancy higher than 80%), and more than 60% of the sites were occupied by an abundance of high mannose glycans (more than 50%). A bNAb binding analysis was also performed and the results illustrated a site-specific correlation of both glycosylation occupancy and class with antigenicity. These initial findings are currently being utilized to facilitate the production of selective antibodies that target specific glycan sites on the Env proteins with the long-term goal of generating more broad-based and effective HIV vaccines.

(P113) A glycan-free Spike protein-based vaccine elicited broad protection against SARS-CoV-2 virus

Kuo-I Lin[1], Szu-Wen Wang[1], Yi-Hsuan Chang[1], Ho-Yang Tsai[1], Xiaorui Chen[1], Che Ma[1], Chi-Huey Wong[1]

[1]Genomics Research Center, Academia Sinica,

We investigated into the adaptive immune responses in mice received a broadly protective vaccine, a mono-GlcNAc-decorated state (S_{MG}) form of Spike protein of SARS-CoV-2. Mice immunized with S_{MG} induced better antibody responses against a broad spectrum of SARS-CoV-2 variants, as compared with that induced by fully glycosylated Spike protein (S_{FG}). Our analysis of IgG subclass titers, and IFN-γ or IL-4 production by T follicular helper (Tfh) cells revealed that S_{MG} vaccine induced higher levels of IgG2a, a more balanced Th1/Th2 response, and more IFN-γ expressing Tfh cells. Furthermore, S_{MG} vaccine induced higher frequency of IL-21^{+} Tfh cells and an elevated level of granzyme B-producing CD8^{+} T cells. These results demonstrated that more potent humoral and cellular adaptive immunity was elicited by a Spike protein with removal of N-glycan. We also examined the frequency and the profiles of Spike protein-specific B cells from the spleen of mice immunized with S_{FG} or S_{MG}, and found that antibodies derived from several specific loci of IGHV and IGKV genes were enriched in the S_{MG} immunized group. A monoclonal antibody, m31A7, from the S_{MG} immunized mice was isolated by single B cell screening platform. m31A7 is able to provide protection against SARS-CoV-2 wild type and variants infection. The cryo–EM structure further revealed the binding of m31A7 to RBD in the "up" state with the N165-glycan from neighboring NTD. Together, our study demonstrated that removal of glycan shields of Spike protein elicited more robust Tfh and CD8 T cell responses against SARS-CoV-2, as well as induced broadly protective antibodies that can efficiently neutralize SARS-CoV-2 variants.

(P114) Glycan composition modulates the antibody-binding affinity and structure of CD16a

Paul Kremer[1], Adam Barb[1,2,3]

[1]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, USA, [2]Department of Chemistry, University of Georgia, Athens, Georgia, USA, [3]Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, USA,

Many therapeutic monoclonal antibodies engage FcγRs including FcγRIIIa/CD16a to stimulate an immune response. CD16a is a heavily glycosylated receptor and its ligand-binding affinity is linked with glycan composition. This suggests that CD16a glycosylation may indirectly play a significant role in the efficacy of therapeutic antibodies. However, the structural mechanism linking glycan composition to affinity remains undefined. We characterized CD16a structure, motion and antibody-binding affinity using a wide distribution of CD16a glycoforms. In agreement with other studies, we found that the removal of terminal sialic acid residues provides the greatest affinity increase compared to removal of any other single sugar residue. We observed stepwise affinity increases with each glycan truncation step up to leaving a single N-acetylglucosamine residue, which showed the highest affinity. These affinity increases only occurred when the N162 glycan was present. We also used NMR spectroscopy to characterize CD16a with three different glycoforms using an innovative protein labeling approach. Residues in the FG loop, which are directly involved in antibody binding, showed large chemical shift perturbations. Other chemical shifts we observed

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mapped to regions proximal to the four other N-glycosylation sites. These data support a model for CD16a where composition of the N162 glycan stabilizes backbone structure near the antibody-binding site.

(P115) Lipoarabinomannan maintains cell wall integrity and regulates cell division and cell envelope elongation in mycobacteria

Ian L. Sparks[1], Japinder Nijjer[2], Jing Yan[2], Yasu Morita[1]

[1]*University of Massachusetts Amherst, MA, USA,* [2]*Yale University, New Haven, CT, USA,*

The growth and division of mycobacteria, including several clinically important pathogens, deviates significantly from that of typical Gram-positive and Gram-negative bacteria. Mycobacteria are rod-shaped and undergo asymmetric polar growth and division in which a diderm envelope is synthesized and elongated from each end of the cell. Furthermore, the old pole elongates faster than the new pole and septa are placed closer to the new pole. The structure and composition of the mycobacterial envelope is as distinctive and unusual as its mode of growth, including many unique molecular components such as the lipoglycans lipomannan (LM) and lipoarabinomannan (LAM). LM and LAM are known to modulate host immunity during infection, but their role outside of intracellular survival remains poorly understood, despite their widespread conservation among non-pathogenic mycobacteria. Previously, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* mutants producing structurally altered LM and LAM were shown to grow slowly in some conditions and to have higher sensitivity to antibiotics, suggesting that these lipoglycans may support cellular integrity or growth. To explore these potential lipoglycan functions in *M. smegmatis*, we tested the effect of multiple LM and LAM biosynthesis mutations on cell envelope integrity, and division. We found that mutants deficient in LAM, but not LM, fail to maintain peptidoglycan cell wall integrity, with envelope deformations specifically associated with septa and new poles, indicating a potential role for LAM in cell growth and division. Further supporting this hypothesis, a mutant producing abnormally large LAM formed multiseptated cells, a characteristic phenotype of mutants with division defects. LAM deficiency was also found to associate with hyper-sensitivity to beta-lactam antibiotics and irregular incorporation of peptidoglycan probes, suggesting a defect specifically in cell wall structure. These results show that regulation of LAM's structure is critical for governing daughter cell separation and peptidoglycan integrity in mycobacteria.

(P116) The biological roles of Bordetella glycans in host-pathogen interaction

Yang Su[1], Barbara Talavera Figueroa[2], Amanda Caulfield[2], Eric Harvill[2], Maor Bar-Peled[1]

[1]*Complex Carbohydrate Research Center, Department of Biochemistry and Molecular Biology, University of Georgia,* [2]*Department of Infectious Disease, University of Georgia,*

Bacterial glycans are critical to maintain cell shape, turgor, survival, and antibiotic resistance. They also play essential roles in virulence, immunomodulation, and host-pathogen interactions. Bacteria belonging to the genus *Bordetella* are known respiratory pathogens, including *Bordetella pertussis*, which infects tens of millions of people and leads to the death of hundreds of thousands of children and infants every year. Other strains infect animals, from dogs, cats, rabbits, and turkeys, to pigs, horses, and seals. *Bordetella* species produce diverse types of glycan structures. However, the biological functions of some glycans remain unclear, especially in the context of colonization, persistence, and transmission. Here, we utilized novel sensitive animal models to distinguish the roles of a few glycans in infections. We discovered a glycan we named b-Cool, bordetellae Colonization Oligosaccharide, that is vital for colonization. The glycan tEPS, transmission-related exopolysaccharide, significantly contributes to transmission. We showed that the tEPS and b-Cool double mutants displayed nearly diminished transmission, indicating the essential roles of both glycans in infections. In addition, we found the lipopolysaccharide O-antigen glycan significantly contributes to virulence. Interestingly, all glycan mutants also displayed increased sensitivity to serum complement. In summary, we have shown that *Bordetella* glycans have significant roles in pathogenicity. Both the tEPS and b-Cool glycans are highly conserved amongst *Bordetella* species, and they are thus potential new vaccine targets to reduce pertussis infection.

(P117) Utilizing the Novel Lectin Abilities of CR3/Mac-1 to Block Neisseria gonorrhoeae

Jessica Poole[1], Jennifer L. Edwards[2,3], Lucy K. Shewell[1], Christopher J. Day[1], Thomas Haselhorst[1], Freda E.-C. Jen[1], Michael P. Jennings[1]

[1]*Institute for Glycomics, Griffith University,* [2]*Center for Microbial Pathogenesis, Abigail Wexner Research Institute at Nationwide Children's Hospital,* [3]*The Department of Pediatrics, The Ohio State University College of Medicine,*

The human pathogen *Neisseria gonorrhoeae* uses pili glycosylated with a terminal galactose to mediate adherence to the I-domain of complement receptor 3 (CR3, CD11b/CD18) expressed on primary human cervical epithelial (pex) cells. This galactose –

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CD11b I-domain interaction is critical to the successful colonization of human cervical cells. Surface plasmon resonance (SPR) was used to characterize the interaction between the I-domain of CD11b and di- or monosaccharide gonococcal pilin. There was no interaction with the monosaccharide modified pilin and the disaccharide pilin, which has a terminal galactose, was determined to have an interaction with recombinant I-domain of CD11b at a K_D (binding affinity) of 349 nM and 907nM with full length CR3. The role of the terminal galactose in this interaction was further verified using commercial glycans. It was found that a terminal galactose with any linkage was sufficient for binding to the I-domain with affinities of the interaction with free glycans ranging 117–320 nM. Using an overlapping peptide library of the I-domain, the area of galactose binding was identified with the peptide G2 able to recapitulate the galactose binding of the full length I-domain. The G2 peptide was then used to block *N. gonorrhoeae* in both CHO-CR3 and pex cell models. A drug screen, performed using SPR with the CD11b I-domain, identified two candidates for drug repurposing: methyldopa (an antihypertensive) and carbamazepine (Cz; an anticonvulsant). Both methyldopa and Cz were found to be able to prevent and treat gonococcal infection in the pex cell model. We then quantified Cz in the serum, saliva, and vaginal fluid collected from 16 women who were, or were not, regularly taking the anticonvulsant. Cz was detected in the vaginal fluid collected from women taking the drug at potentially therapeutic levels using a competitive ELISA. Furthermore, the Cz concentrations present in the vaginal fluid was sufficient to result in a greater than 99% reduction (within 24 h) in the number of viable gonococci recovered from the pex cell model. These data provide evidence that disrupting the interaction between the terminal galactose on gonococcal pilin and the CD11b I-domain using methyldopa or Cz may act as a novel, host-targeted therapy to treat gonococcal cervicitis.

(P118) Characterising glycan recognition in the cholesterol-dependant cytolysin Suilysin of Streptococcus suis

Lucy K. Shewell[1], Greg Tram[1], John M. Atack[1], Thomas Haselhorst[1], Freda E.C. Jen[1], Jessica Poole[1], Christopher J. Day[1], Michael P. Jennings[1]

[1]*Institute for Glycomics, Griffith University, Gold Coast, Queensland, 4222, Australia,*

Streptococcus suis is a pathogen that primarily affects swine herds and causes respiratory and invasive disease. *S. suis* is also a zoonotic pathogen with cases of human disease reported in southeast Asia, Europe and the Americas. Pathogenesis by *S. suis* is complex and involves many virulence factors. Disease begins with colonisation of the respiratory system that can progress to systemic invasion and a subsequent invasion of the CNS. Many virulent *S. suis* strains express a toxin known as Suilysin (SLY), which is suggested to play a role in the progression of deep tissue infections and modulating the host immune response.

SLY belongs to the cholesterol-dependant cytolysin (CDCs) family. These are pore forming toxins expressed by a range of gram-positive bacterial species. CDCs are secreted as soluble monomers which assemble into an oligomeric complex on the target cell membrane. This binding to host cells was initially thought to be solely dependent on cholesterol; however, our group has previously demonstrated that all CDCs also utilise host carbohydrates as cellular receptors. SLY can engage with three distinct glycan structures as cellular receptors; αGal/Galili epitope, Blood Group A and the P1 antigen. Molecular modelling and NMR-CSP studies were used to characterise the binding sites for each of the three identified glycan receptors for SLY. Structure guided site-directed mutagenesis was used to further map the residues in SLY required for carbohydrate recognition. Generated SLY mutants were then screened for loss of glycan binding using surface plasmon resonance and assessed for hemolytic activity. We demonstrate three distinct lectin sites at the base of SLY domain 4, a region that interacts with the cell membrane, consistent with a role in receptor recognition. This research significantly advances our understanding of carbohydrate recognition in SLY and provides valuable insight into the cellular interactions shared across all CDCs.

(P119) The protective effect of a recombinant protein vaccine directed against HSV-2 is highly dependent on the incidence of glycan structures

Ebba Samuelsson[1], Carolina Gustafsson[1], Ekaterina Migorodskaya[2], Jonas Nilsson[2], Staffan Görander[1], Jan-Åke Liljeqvist[1], Rickard Nordén[1]

[1]*Section of Virology, Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden,* [2]*Proteomics Core Facility, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden,*

Herpes Simplex Type 2 (HSV-2) constitutes a global problem with an estimated 490 million individuals infected worldwide, still no vaccines are approved for commercial use. Here, we have used a recombinant protein vaccine candidate based on the viral glycoprotein gG2. We defined the glycan composition and assessed the impact of glycosylation on the protective effect in a murine model.Using LC MS/MS we defined all glycan sites and glycoform distribution of the recombinant gG2, identifying two N-linked glycans of primarily complex- and high mannose type and eleven core 1 O-linked glycans, extensively decorated with sialic acids. To assess the protective effect, we modulated the glycan content of the recombinant gG2, obtaining a total of five

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separate vaccine candidates with specific glycosylation profiles. The protective effect, of all the vaccine candidates, was assessed in a mouse model, along with measurements of antibody production and antibody reactivity. Recombinantly expressed gG2 with intact glycosylation profile showed 92.3% protection (24/26 mice survived viral challenge) when mice were challenged with a lethal dose of HSV-2 strain 333. Removal of both O- and N-linked glycans from the vaccine reduced the protection with 48.5% (7/16 mice survived viral challenge, $p = 0.0025$). Removal of distinct types of glycan structures or only the terminating sialic acid of the glycan chains conferred a marginal reduction in protection. An intact glycosylation profile was essential for protection, but the induced anti-gG2 IgG levels were similar for all vaccine candidates. However, antibody recognition of the fully glycosylated gG2 was reduced in sera from mice that received the completely deglycosylated vaccine candidate ($p = 0.0026$). This indicates that immunization with gG2 devoid of glycan structures generates an antibody pool that fail to bind to fully glycosylated gG2, possibly due to glycan shielding of potent antibody epitopes. Infectious HSV-2 virus particles contain glycoproteins which are densely glycosylated. Thus, a possible explanation to the reduced protective effect of the deglycosylated vaccine candidate would be that the antibody pool is skewed towards epitopes that are not accessible in the fully glycosylated vaccine candidate or the infectious viral particles. Another hypothesis could be that certain glycan structures together with the peptide backbone constitute antibody epitopes that are necessary for eliciting a proper protective response.

(P120) MGAT1 knockout in MUTZ-3 dendritic cells lead to increased expression of MHC class I/II and increased CD8+ T cell activation

Sarah Line Skovbakke[1,†], Anne Louise Blomberg[1,†], Weihua Tian[1], Steffen Goletz[1]

[1]*Department of Biotechnology and Biomedicine, Section for Protein Science and Biotherapeutics, Biotherapeutic Glycoengineering and Immunology, Technical University of Denmark,*

† Co-first authors

Dendritic cells (DC) are specialized immune cells that play a crucial role in shaping the immune response towards tolerance or inflammation, making them an interesting target for many therapeutic approaches[1]. Previous studies have linked cell surface sialylation with DC function using gene expression profiling of relevant sialyltransferases, as well as removal or blocking of cell surface sialylation using sialidases and metabolic inhibition of sialylation[2–5]. However, the role of many glycosyltransferases in DC biology has still not been dissected and functional studies of how specific glycosylation pathways and structures regulate human DC biology, have been hampered by the lack of suitable cell systems. Therefore, we recently developed a strategy to genetically glycoengineer the human myeloid leukemia cell line MUTZ-3, the currently best cell-line-based model for human DCs[6], and used this model to study the role of *ST6GAL1* and the resulting α2,6-linked sialylation in DC biology[7].

In the current study, we used this method to obtain knockout (KO) of MGAT1 in MUTZ-3 cells (confirmed through Sanger sequencing and lectin staining) enabling investigation of how a switch from complex to oligomannose N-glycans affects DC biology.

Our data show that *MGAT1* $^{-/-}$ precursor cells efficiently differentiate into immature dendritic cells (iDCs) confirmed by expression of DC surface markers including CCR6, CD1a, CD80, CD86, and CD209. Interestingly HLA-DR was significantly higher in *MGAT1*$^{-/-}$ iDCs compared to WT and the same tendency was observed with HLA-ABC. Upon TNFα induced maturation of iDCs the difference in HLA-DR and HLA-ABC was even more profound ($p<0.00001$, $p=0.0069$ respectively). Furthermore, a 5 day coculture of *MGAT1*$^{-/-}$ iDCs and T cells from healthy donors resulted in greater CD8$^+$ T cell activation (surface CD25 expression and proliferation) compared to WT ($p=0.001$). No difference was observed on CD4$^+$ T cell activation.

These results indicate that the structure of N-linked glycans regulates MHC expression in human DCs as well as DC-induced activation of CD8$^+$ T cells, and that KO of *MGAT1* may improve the immunogenic properties of DCs. Further investigations are needed to dissect the mechanism of N-glycan-induced regulation in DC biology and DC-T cell interactions.

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(P121) Improving activation of CD4+ T cells through knocking out ST6GAL1 in MUTZ-3 dendritic cells

Weihua Tian[1,*], Anne Louise Blomberg[1,*], Sarah Line Skovbakke[1,†], Steffen Goletz[1,†]

[1]*Biotherapeutic Glycoengineering and Immunology, Section for Protein Science and Biotherapeutics, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, 2800 Kgs Lyngby, Denmark,*

*Co-first authors
‡Co-corresponding authors

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Dendritic cells (DCs) are specialized immune cells that activate T cells by presenting antigens on their surface. This activation is essential for launching effective immune responses against pathogens and coordinating various immune functions[1]. How distinct glycan structures on DCs contribute to T cell activation remains inadequately understood, partly due to a lack of appropriate models[2]. Building upon our recent success in establishing the *ST6GAL1* knockout (KO) within the human myeloid leukemia cell line MUTZ-3[3], here we aim to investigate how modifications of 2,6-linked sialylation on MUTZ-3 cells influence the T cell activation process.

In our previous work, we showed that *ST6GAL1*$^{-/-}$ iDCs express more antigen-presenting molecules and co-stimulatory factors[3], which could make it more immunogenic and more effective in activating T cells. To investigate this hypothesis, we conducted cocultures involving both wild-type (WT) and *ST6GAL1*$^{-/-}$ iDCs with primary human T cells over a five-day period. Subsequently, we assessed T-cell stimulation by examining the cell-surface expression of activation-related receptors (CD69 and CD25) and T cell proliferation using flow cytometry. Sub-gating on $CD4^+$ and $CD8^+$ T cells unveiled elevated surface expression of CD69 in $CD4^+$ T cells following co-culture with *ST6GAL1*$^{-/-}$ iDCs compared to WT, indicating a higher level of helper T (TH) -cell activation due to the absence of 2,6-linked sialylation. Additionally, we quantified the concentrations of various cytokines and chemokines in the culture media via mesoscale multiplex assays. Specifically, we observed significant increases in the levels of key immune mediators, including IFNγ, IL-2, TNFβ, IL-13, IL-5, IL-10, CCL22, and CCL17, after T-cell coculture with *ST6GAL1*$^{-/-}$ iDCs as compared to their WT counterparts.

In summary, these discoveries showed that *ST6GAL1*$^{-/-}$ iDCs enhances the activation of $CD4^+$ T cells, resulting in an elevated expression of CD69 and cytokine secretion. Furthermore, these findings highlight the promising therapeutic possibilities of modulating immune responses through modifying sialylation pathways in DC.

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(P122) Hyaluronidase in *Streptococcus pyogenes* – analysis based on structural biology and molecular phylogenetics

Kotaro Higashi[1], Masaya Yamaguchi[1,3,4,5], Katsuki Takebe[6], Masanobu Nakata[7], Tomoko Sumitomo[8], Mamoru Suzuki[9], Victor Nizet[10,11,12], Shigetada Kawabata[1,5]

[1]Department of Microbiology, Graduate School of Dentistry, Osaka University, Osaka, Japan, [2]Department of Removable Prosthodontics and Gerodontology, Graduate School of Dentistry, Osaka University, Osaka, Japan, [3]Bioinformatics Research Unit, Graduate School of Dentistry, Osaka University, Osaka, Japan, [4]Bioinformatics Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, [5]Center for Infectious Disease Education and Research, Osaka University, Osaka, Japan, [6]Department of Oral & Maxillofacial Oncology and Surgery, Graduate School of Dentistry, Osaka University, Osaka, Japan, [7]Department of Oral Microbiology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan [8]Department of Oral Microbiology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima, Japan, [9]Laboratory of Supramolecular Crystallography, Institute for Protein Research, Osaka University, Osaka, Japan, [10]Department of Pediatrics, School of Medicine, University of California San Diego, San Diego, CA, USA, [11]Division of Host-Microbe Systems & Therapeutics, University of California San Diego, San Diego, CA, USA, [12]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, San Diego, CA, USA,

Some bacteria rely on hyaluronidases for survival, enabling them to evade host immunity and acquire carbon sources for growth. The Gram-positive human-specific pathogen *Streptococcus pyogenes* is associated with a broad spectrum of infectious diseases, ranging from mild to severe invasive conditions. *S. pyogenes* has been classified into at least 240 different types based on genotyping of the *emm* gene, which encodes the M protein, a major virulence factor. While most *emm*-type *S. pyogenes* possess a hyaluronic acid (HA) surface capsule and an inactive hyaluronidase (HylA) enzyme, some, like *emm*4 strains, lack HA capsule and carry a V199D polymorphism in HylA that restores hyaluronidase activity. This present study investigated the phylogenetic relationship, crystal structure, and potential function of *S. pyogenes* HylA applying computational protein science methods. A Bayesian phylogenetic tree constructed using *hylA* gene sequences indicated that active HylA in *S. pyogenes* evolved from inactive HylA due to natural mutations over time. Crystal structure analysis explored the effects of a point mutation in HylA on protein structure. Recombinant inactive HylA from *emm*1 *S. pyogenes* (M1HylA) was crystallized and its structure determined at a resolution of 2.4 Å. We observed that the region between the $\alpha 4$ and $\alpha 5$ helices, which contains V199, was a disordered long loop region, with V199 not acting as an active residue but rather indirectly causing inactivation. Thus a single point mutation reduces HylA structural stability and alters the catalytic site structure. Further molecular dynamics simulation for M1HylA was performed using GROMACS. The root mean square fluctuation analysis results showed that the region of high flexibility coincided with the disordered region of the M1HylA crystal structure, while root mean square deviation (RMSD) analysis indicated that the domain containing V199 had a higher RMSD than the other domain. Thus V199 could be responsible for the structural instability. And while reduced stability of active residues eliminates hyaluronidase activity, it remains unclear whether M1HylA retains the ability to bind hyaluronan. Our prediction of the molecular interaction between HylA and hyaluronan performed using AutoDock Vina yielded complex models and affinity calculations suggesting the possibility that inactive HylA retains its hyaluronan binding ability.

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Collectively, these results highlight that a point mutation outside the active site of HylA can alter the secondary structure and hyaluronidase activity. Conversely, the binding region was found to be structurally conserved, suggesting that the hyaluronan-binding ability is retained. This computational approach supports prior hypotheses derived from experimental results.

(P123) Differences in Human and Mouse Immunological Evaluation of Cancer Vaccines Based on Ganglioside Antigens and α-Galactosylceramide

Sarah Line Skovbakke[1], Anne Louise Blomberg[1], Lise Hafkenscheid[1], Cecilia Romano[2], Hao Jiang[2], Sahar Tahvili[2], Peng Wei[2], Jonas R. Henriksen[3], Anders E. Hansen[3], Mads H. Clausen[2], Steffen Goletz[1]

[1]Department of Biotechnology and Biomedicine, Section for Protein Science and Biotherapeutics, Biotherapeutic Glycoengineering and Immunology, Technical University of Denmark, Søltofts plads, 2800 Kgs Lyngby, Denmark, [2]Center for Nanomedicine & Theranostics, Department of Chemistry, Technical University of Denmark, Kemitorvet 207, 2800 Kgs. Lyngby, Denmark, [3]Department of Health Technology, Section for Biotherapeutic Engineering and Drug Targeting, Technical University of Denmark, Ørsteds Plads, 2800 Kgs Lyngby, Denmark,

iNKT cells have, due to their unique properties at the interphase between innate and adaptive immunity, emerged as central players in cancer vaccine therapies. iNKT cells orchestrate immune responses via the recognition of glycolipids presented by the non-polymorphic MHC class I-like CD1d protein expressed by antigen presenting cells (APCs) such as dendritic cells (DCs). Glycolipids activating iNKT cells, such as α-galactosylceramide (αGalCer), can enhance the immune response against co-delivered cancer antigens and have been applied in the design of self-adjuvanting anti-tumor vaccines.[1–3] Surface expression of the GM3 ganglioside is higher in cancers in comparison to normal tissues.[4] More interestingly, the structurally similar (Neu5Gc)GM3 ganglioside is almost undetectable in normal human tissues, but highly expressed in several human cancer cells,[5] making it an excellent target for cancer vaccine development.[6,7] Therefore, we aimed to synthesize GM3 and (Neu5Gc)GM3 conjugates with αGalCer, to obtain fully synthetic vaccine constructs formulated in liposomes, for efficient co-delivery of antigen and adjuvant to the same APC.

Herein we present a comparison of stimulation of mouse and human DCs with liposomes containing (Neu5Gc)GM3- and GM3-αGalCer conjugates as well as equimolar amounts of the two. *In vitro* stimulation of human DCs with the liposomes selectively induces CD1d expression and iNKT cell expansion upon coculture with a mixture of human $CD3^+$ lymphocytes. Furthermore, mouse BMDCs loaded with the liposomes induced TH1 and TH2 cytokine secretion *in vitro* upon coculture with isolated iNKT cells, while *in vivo* administration induced expansion of splenic iNKT cells and increased serum cytokine levels. Interestingly, liposomes containing equimolar amounts of (Neu5Gc)GM3- and GM3-αGalCer conjugates showed the highest expansion of human iNKT cells while the opposite trend was observed in the mouse system. These findings warrant further investigation of the difference between human and mouse iNKT cells and highlights the importance of human test systems for translation of iNKT cell targeting glycolipid cancer vaccines.

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(P124) Endothelial glycocalyx injury as a risk factor for severe acute kidney injury and disseminated intravascular coagulation development in patients with urological sepsis

Naoki Fujita[1], Yuki Tobisawa[2], Tohru Yoneyama[3], Shingo Hatakeyama[4], Takahiro Yoneyama[5], Yasuhiro Hashimoto[1], Chikara Ohyama[1,5]

[1]Department of Urology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan, [2]Department of Urology, Gifu University Graduate School of Medicine, Gifu, Japan, [3]Department of Glycotechnology, Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine, Hirosaki, Japan, [4]Department of Advanced Blood Purification Therapy, Hirosaki University Graduate School of Medicine, Hirosaki, Japan, [5]Department of Advanced Transplant and Regenerative Medicine, Hirosaki University Graduate School of Medicine, Hirosaki, Japan,

Introduction: The glycocalyx covers the luminal surface of vascular endothelial cells and has an important role in thrombogenicity, inflammation, and vascular permeability. Severe acute kidney injury (AKI) and disseminated intravascular coagulation (DIC) are common complications in patients with urological sepsis. However, the association between endothelial glycocalyx injury and severe AKI and DIC development in urological sepsis remains unclear.

Methods: This retrospective study included 140 patients with urological sepsis treated between March 2017 and March 2019. AKI was defined according to the KDIGO criteria. Plasma syndecan-1 and hyaluronan levels at the admission were measured by enzyme-linked immunosorbent assay. Multivariable logistic regression analyses were performed to evaluate the association between glycocalyx injury and severe AKI and DIC development. Moreover, we evaluated the predictive ability of elevated

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syndecan-1 and hyaluronan levels for severe AKI and DIC development using the receiver operating characteristic curve and compared using net reclassification improvement and integrated discrimination improvement.
Results: Median age was 79 years old in this cohort. Of the 140 patients, 37 and 24 developed severe AKI (AKI stage 2–3) and DIC, respectively. Plasma syndecan-1 and hyaluronan levels were significantly correlated with specific markers of thrombin generation, such as soluble fibrin ($P = 0.007$ and $P = 0.020$, respectively) and prothrombin fragment 1+2 ($P < 0.001$ and $P = 0.024$, respectively). Patients who developed severe AKI had higher plasma syndecan-1 and hyaluronan levels than those who did not ($P < 0.001$ and $P < 0.001$, respectively). Similarly, patients who developed DIC had higher plasma syndecan-1 and hyaluronan levels than those who did not ($P < 0.001$ and $P < 0.001$, respectively). In multivariable analyses, plasma syndecan-1 and hyaluronan levels were selected as a significant risk factor for severe AKI ($P = 0.036$ and odds ratio [OR] 2.700, $P = 0.005$ and OR 3.545, respectively) and DIC ($P < 0.001$ and OR 16.47, $P < 0.001$ and OR 6.023, respectively) development. Elevated plasma syndecan-1 and hyaluronan levels improved predictive abilities for severe AKI and DIC development compared to other well-known risk factors alone.
Conclusion: Endothelial glycocalyx injury was a risk factor for severe AKI and DIC development in patients with urological sepsis. Endothelial glycocalyx injury may be a potential therapeutic target for severe AKI and DIC.

(P125) Transcriptional upregulation of host gene glycosyltransferase FUT6 in Tick-borne encephalitis virus infected T-cells

Sofia Brunet[1], Arman Bahari[1], Kristina Nyström[1]

[1]*Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden,*

Tick-borne encephalitis virus (TBEV) is a Flavivirus that is endemic in large areas in central Europe, Scandinavia, and Asia. The virus is spread through bite of an infected tick or consumption of unpasteurized dairy products. Infection can cause severe central nervous system (CNS) involvement, with permanent neurological complications or in critical cases death. Several strategies for TBEV to cross the blood-brain barrier have been suggested. In the "Trojan horse" hypothesis, infected immune cells are hijacked by the virus to migrate across the epithelial cells into the CNS. In this work, we investigate the ability of TBEV to stimulate activation of circulating leukocytes by upregulation of glycosyltransferases involved in synthesis of sialyl Lewis X (sLeX). The human H9 cell line was used as a model for CD4+ T-cell infection. RNA from infected cells were analysed using qPCR och gene expression of human FUT gene, encoding fucosyl-transferases determined. Infected H9 cells showed a 1.053 log unit increase of FUT6 expression, 72 hours post infection in comparison to mock control (p-value 0.01). There was not a significant change in expression in any of the other genes encoding fucosyltransferases tested (FUT1, 3, 5, 7). As each cell type have its own glycosylation machinery, we compared the T-cell findings with TBEV infection of the human lung epithelial cell line A549. In contrast to the H9, FUT expression did not differ between the infected cells and mock control. FUT6 encodes the fucosyltransferase VI (Fuc-TVI), responsible of adding a fucose in the α(1-3) linkage in the last step of sLeX synthesis. Upon T-cell activation, sLeX expression is induced, enabling the cell to bind to P- and E-selectin on the vascular endothelium for leukocyte extravasation. It has previously been demonstrated that herpesviruses upregulate expression of fucosylated glycans on the cell surface of infected T-cells. Our results support this and indicates that TBEV induces activation of T-cells to enhance spread from the blood to the CNS.

(P126) Use of Siglec 2-targeting platforms for the induction of protective tolerance

Katarzyna A. Brzezicka[1,2], Britni M. Arlian[1,2], Maidul Islam[1,2], Shrawan Chavan[1,2], James C. Paulson[1,2]

[1]*Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA,* [2]*Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, USA,*

Unwanted immune responses are the common cause of several pathological conditions, such as autoimmune diseases. They can be also mounted in response to the drug administration, causing the formation of anti-drug antibodies (ADAs) affecting the drug's activity and efficacy. In recent years Siglecs, sialic-acid-binding immunoglobulin (Ig)-like lectins, have gained attention as immune checkpoints for therapeutic interventions to dampen excessive immune responses and to restore immune tolerance. Many Siglecs, including Siglec 2, function as inhibitory receptors suppressing activation signals in various immune cells through binding to sialic acid ligands and thus could become an excellent target to control autoimmunity or ADAs formation.

Here we report the development of two different platforms targeting B cell inhibitory co-receptor CD22 modulating immune responses. First, CD22 ligand (CD22L) will be displayed on the surface of hybrid nanoparticles (NPs) composed of a PLGA core coated with a lipid monolayer. CD22L-NPs will also display the antigen of interest and will encapsulate the immunosuppressive drug rapamycin which will help to induce antigen-specific tolerance in both T cells and B cells. In the K/BxN mouse model of rheumatoid arthritis caused by B and T cell-dependent responses to the self-antigen glucose-6-phosphate-isomerase (GPI), we

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show that CD22L/GPI nanoparticles delay the development of disease, with some treated mice remaining arthritis-free for 300 days.

The second strategy involves coupling the CD22 ligand directly to a protein of interest. We have devised a simple conjugation strategy for the conjugation of CD22L, and when conjugated to an exemplary immunogenic protein, anti-program cell death protein 1 (anti-PD1) can dramatically suppress antibody production *in vivo* compared antibodies produced to unconjugated anti-PD1.

The results show the potential of different Siglec 2 targeting platforms for inducing immune tolerance to self and non-self-antigens for suppressing autoimmune diseases or ADAs to immunogenic biotherapeutic proteins.

(P127) IgG variable region sialic acid deacetylation enables antibody protection against intracellular infection

John Erickson[1], Stephanie Archer-Hartmann[3], Alexander E. Yarawsky[4], Jeanette L.C. Miller[4], Stephanie Seveau[5], Yuehong Wu[6], Brian R. Wasik[7], Colin R. Parrish[7], Yueh-Chiang Hu[8], Joseph T.Y. Lau[9], Parastoo Azadi[3], Andrew B. Herr[4], Sing Sing Way[2,6]

[1]Division of Neonatology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [2]Center for Inflammation and Tolerance, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA, [4]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [5]Department of Microbial Infection and Immunity, Ohio State University, Columbus, OH, USA, [6]Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [7]College of Veterinary Medicine, Department of Microbiology and Immunology, Cornell University, Ithaca, NY [8]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [9]Department of Molecular and Cell Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY,

Antibodies have long been thought to poorly defend against infections caused by intracellular pathogens, creating a potential gap in immunity for neonates who rely on vertically transferred maternal IgG for early life host defense. Ongoing work in our laboratory shows that pregnancy-induced modification of antibody N-glycans enables protection against the prototypical intracellular pathogen *Listeria monocytogenes* (Lm). Infection susceptibility is overturned in neonatal mice born to preconceptually primed mothers possessing Lm-specific IgG or upon passive transfer of antibodies from primed pregnant, but not virgin, mice. Maternal immunization targeting a single Lm virulence factor, listeriolysin O, recapitulates this robust protection against neonatal infection. Using a precise glycoengineering approach, we demonstrate that N-linked glycan terminal sialic acid residues on IgG from Lm immunized pregnant mice are critical for protection. Replacement of the existing sialic acid on IgG from virgin mice with either Neu5Ac or Neu5Gc enables protection, whereas replacement with 9-O-acetylated sialic acid results in non-protective antibodies, highlighting discordant *in vivo* antibody defense is modulated by a single acetyl group on sialic acid. Importantly, sialic acid acetyl esterase (SIAE) is upregulated during pregnancy and required to deacetylate terminal sialic acid residues on IgG Fab variable region N-linked glycans. SIAE enzymatic treatment of $F(ab')_2$ fragments from Lm immune virgin mice is sufficient to enable protection. Deacetylated Lm-specific IgG protects neonates via the sialic acid receptor CD22, which suppresses B cell IL-10 production to unleash antibody-mediated protection. Consideration of the maternal-fetal dyad as a joined immunological unit unveils newfound protective roles for antibodies and fine-tuned adaptations to enhance host defense during pregnancy and early life, with broader implications for vaccines and therapeutics targeting intracellular infections.

(P128) Fucosylated glycoproteins and fucosylated glycolipids play opposing roles in cholera intoxication

Atossa C. Ghorashi[1], Andrew Boucher[2], Stephanie A. Archer-Hartmann[3], Nathan B. Murray[3], Rohit Sai Reddy Konada[1], Xunshi Zhang[4], Chao Xing[4,5], Parastoo Azadi[3], Ulf Yrlid[2], Jennifer Kohler[1]

[1]Department of Biochemistry, UT Southwestern Medical Center, Dallas TX 75390 USA, [2]Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, 405 30 Gothenburg, Sweden, [3]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Road, Athens, GA 30602, USA, [4]McDermott Center for Human Growth and Development, UT Southwestern Medical Center, Dallas TX 75390 USA, [5]Department of Bioinformatics, UT Southwestern Medical Center, Dallas TX 75390 USA,

Cholera toxin (CT) is the etiological agent of cholera. Here we report that multiple classes of fucosylated glycoconjugates function in CT binding and intoxication of intestinal epithelial cells. In Colo205 cells, knockout of B3GNT5, the enzyme required for synthesis of lacto- and neolacto-series glycosphingolipids (GSLs), reduces CT binding but sensitizes cells to intoxication. Overexpressing B3GNT5 to generate more fucosylated GSLs confers protection against intoxication, indicating that fucosylated

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GSLs act as decoy receptors for CT. Knockout (KO) of B3GALT5 causes increased production of fucosylated O-linked and N-linked glycoproteins, and leads to increased CT binding and intoxication. Knockout of B3GNT5 in B3GALT5 KO cells eliminates production of fucosylated GSLs but increases intoxication, identifying fucosylated glycoproteins as functional receptors for CT. These findings provide insight into molecular determinants regulating CT sensitivity of host cells.

(P129) Aberrantly O-glycosylated IgA1 in the pathogenic circulating immune complexes in patients with IgA nephropathy is associated with IgG and complement C3

Stacy Hall[1], Graham Gurganus[1], Zhi-Qiang Huang[1], Nicolas Maillard[2], Zina Moldoveanu[1], Dana V. Rizk[1], Bruce A. Julian[1], Matthew B. Renfrow[1], Jan Novak[1]

[1]*University of Alabama at Birmingham, Birmingham, AL, USA,* [2]*Hospital and University Jean Monnet of Saint-Etienne, Saint-Etienne, France,*

IgA nephropathy (IgAN) is an autoimmune kidney disease wherein circulating immune complexes (CIC) deposit in the glomeruli and induce mesangioproliferative injury. These CIC contain IgA1 with some *O*-glycans deficient in galactose (Gd-IgA1) bound by IgG autoantibodies specific for Gd-IgA1. Serum levels of Gd-IgA1 and IgG autoantibodies predict disease progression, but little is known about the corresponding CIC. We analyzed the mesangioproliferative capacity and composition of IgA1-containing CIC from patients with progressive vs. non-progressive IgAN.

CIC were isolated from sera of IgAN patients with progressive (IgAN-P; n=3) and non-progressive (IgAN-Np; n=2) disease using size-exclusion chromatography (SEC). IgAN-P had eGFR loss >2 ml/min/1.73 m^2 per year. Mesangioproliferative activities of the individual fractions of CIC of molecular mass (Mr) 700–2,000 kDa and pooled fractions corresponding to pooled CIC of high (CIC-0), medium (CIC-1), and low (CIC-2) Mr were assessed using cultured primary human mesangial cells (MC). Serum depleted of IgA1 by jacalin affinity chromatography served as a negative control. Serum levels of IgA and Gd-IgA1 were determined by ELISA. Composition of CIC was assessed by SDS-PAGE/immunoblotting, using either a method that preserves the C3 thioester and disulfide bonds, or under reducing conditions that break disulfide and thioester bonds. Immunoblots were probed with antibodies specific for IgA, IgG, or complement C3. Purified IgA1, IgG, C3, C3b, and iC3b served as standards. Under reducing conditions, immunoblotting for C3 showed, in addition to b chain, a and a' chains of C3 and C3b, respectively, and a 47-kDa band of iC3b.

MC proliferation was stimulated by the SEC-fractionated CIC of Mr 700–2,000 kDa. Pooled CIC of medium and low Mr, i.e., CIC-1 and CIC-2, exhibited the most stimulatory activity, whereas pooled CIC of high Mr, i.e., CIC-0, had little activity. CIC-1 and CIC-2 from IgAN-P exhibited greater mesangioproliferative activity compared to those from IgAN-Np. Jacalin affinity chromatography removed the stimulatory activity from CIC-1 and CIC-2, but not from CIC-0. These stimulatory CIC-1 and CIC-2 contained IgA, IgG, and C3. IgA in these CIC was predominantly polymeric IgA1, enriched for galactose-deficient and minimally sialylated *O*-glycoforms. C3 in CIC was covalently associated with IgG and IgA1, presumably through the thioester bond. Immunoblotting of CIC electrophoretically separated under reducing conditions revealed that IgAN-P had more C3 and C3b, whereas CIC of IgAN-Np had more iC3b. Jacalin affinity chromatography depleted IgA1, IgG, and C3 from CIC-1 and CIC-2 and removed their stimulatory activity.

In patients with IgAN, IgA1-containing CIC of medium and low Mr stimulated proliferation of MC *in vitro*. These CIC consisted of Gd-IgA1, IgG, and C3. C3 was covalently attached to IgA1 and IgG. Future studies will determine molecular characteristics of CIC associated with disease progression.

(P130) FcRn Mediates IgG Sialylation in Endothelial Cells

Leandre Glendenning[1], Kalob Reynero[1], Emily Kukan[1], Megan Long[1], Brian Cobb[1]

[1]*Department of Pathology, Case Western Reserve University,*

Sialylation of the conserved glycan on the Fc domain of IgG comprises a key "inflammatory switch" in the host immune response. For decades, it has been well established that decreases in IgG Fc sialylation strongly correlate with various inflammatory conditions, ranging from infections such as HIV and tuberculosis to chronic autoimmune disorders such as rheumatoid arthritis. Despite the abundance of evidence that IgG sialylation is tightly regulated in disease pathogenesis, the mechanisms underlying the transition from asialylated to sialylated IgG remain uncertain. Conventionally, it has been presumed that IgG sialylation occurs in the *trans*-Golgi network of antibody-producing plasma cells; however, we demonstrated in 2016 that mice lacking ST6Gal1 in the B cell compartment have unaltered plasma IgG sialylation. Further, we have shown that B cells produce largely asialylated IgG, leading us to conclude that IgG sialylation is largely regulated following its secretion into the plasma by the B cells. Here, we report that isolated endothelial cells are capable of sialylating IgG in the culture supernatant. This effect was not seen in endothelial cells isolated from FcRn-deficient mice, suggesting that FcRn is essential for regulating IgG sialylation. Murine bone marrow-derived macrophages, which also abundantly express FcRn, failed to sialylate IgG, suggesting that FcRn is not sufficient

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for IgG sialylation. Building on our previous studies indicating the importance of intracellular ST6Gal1 localization, we further confirmed that IgG is co-localizing with ST6Gal1 inside of endothelial cells. Together, these data support the existence of a novel FcRn-mediated glycan remodeling pathway in endothelial cells which drives B cell-extrinsic IgG sialylation.

(P131) M2 macrophages exhibit enhanced environmental sialylated glycan sensing via Siglec-2

Emily Kukan[1], Kevin Telfer[1], Julie Zhou[1], Corwin Nycholat[2], James Paulson[2], Brian Cobb[1]

[1]*Department of Pathology, Case Western Reserve University School of Medicine,* [2]*Department of Molecular Medicine, The Scripps Research Institute,*

Macrophages exhibit a diverse spectrum of phenotypes and functions ranging from classically-activated, pro-inflammatory M1 cells to alternatively-activated, anti-inflammatory M2 cells. Moreover, this phenotype can be influenced by the local microenvironment of the tissues in which macrophages reside. In tissues like the liver and lung that experience a greater deal of antigen exposure, tissue-resident macrophages skew toward an M2-like phenotype, exhibiting a higher activation threshold and displaying more anti-inflammatory functions. Similarly, glycans are well-known to influence the phenotypes and functions of cells, and glycan profiles vary between different tissues. Thus, we aimed to study the influences that tissue glycans can exert on macrophages. To do so, we used an existing mouse in our lab, a hepatocyte-specific conditional knockout of ST6Gal1 (HcKO) which lacks α2,6-linked sialic acids on its hepatocytes. The macrophage population in the liver of this mouse showed a shift away from the normal M2 anti-inflammatory phenotype to a pro-inflammatory M1 phenotype. To better understand the relationship between α2,6-linked sialic acids and macrophages, we turned our attention to Siglec 2 (CD22), a sialic acid-binding Ig-like lectin which binds exclusively to α2,6-linked sialic acids. CD22 has been extensively characterized as a B cell marker, exhibiting inhibitory effects upon ligation that dampen B cell signaling through the recruitment of phosphatases. We have shown that M0 macrophages modestly express CD22, but M2 macrophages show robust upregulation of CD22. Further, CD22 knockout macrophages show increased expression of pro-inflammatory cytokines upon challenge with HMGB1, a toll-like receptor 4 agonist. Our ongoing work focuses on determining the function of CD22 in macrophages and understanding the relationship between this receptor, environmental α2,6-linked sialic acids, and the effector functions of macrophages.

(P132) The structure of a C. neoformans GXM polysaccharide recognized by protective antibodies: A combined NMR and MD study

Audra Hargett[1], Hugo Azurmendi[1], Conor Crawford[2,3], Maggie Wear[2], Stefan Oscarson[3], Arturo Casadevall[2], Daron Freedberg[1]

[1]*Laboratory of Bacterial Polysaccharides, Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA,* [2]*W. Harry Feinstone Department of Molecular Microbiology and Immunology, John Hopkins Bloomberg School of Public Health, Baltimore, MD, USA,* [3]*Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland,*

Cryptococcus neoformans is a fungal pathogen responsible for cryptococcosis and cryptococcal meningitis. The *C. neoformans* capsular polysaccharide and shed exopolysaccharide function both as key virulence factors and to protect the fungal cell from phagocytosis. Currently, a glycoconjugate of these polysaccharides (PS) is being explored as a vaccine to protect against *C. neoformans* infection. To determine the smallest oligosaccharide unit necessary to bind to anti-GXM monoclonal antibodies (mAbs), a library of twenty-six synthetic GXM oligosaccharides was tested against a mAb panel. An *O*-acetylated GXM decasaccharide (GXM10-Ac_3) consisting of an extended M2 motif, which is common in the clinically predominant serotype A strains, is the smallest unit that binds strongly to most anti-GXM mAbs. The M2 motif is characterized by a 6-residue α-mannan backbone repeating unit, consisting of a triad of α-(1➔3)-mannoses (Man), modified by β-(1➔2)-xyloses (Xyl) on the first two Mans and a β-(1➔2)-glucuronic acid (GlcA) on the third Man.

In this study, we utilize modern NMR methods to enhance signal resolution and higher fields than previously utilized to thoroughly characterize the GXM10-Ac_3 PS. We only observed one very weak inter-residue NOE peak that was not across a transglycosidic bond, supporting an extended structure model. To complement the NMR data, a 2 μs GXM10-Ac_3 MD simulation was collected, and the average structure obtained from the MD simulation agree with the NMR NOE and *J*-coupling data. GXM10-Ac_3 f and y transglycosidic torsion analysis indicate that the Man backbone bond flexibility is limited by the branched glycans while the *O*-acetylation has no effect on the range of motion. The Man-Xyl/GlcA transglycosidic torsion angle population analysis indicates that these branches exhibit a broad range of motion with two or more local energy minima. Overall, these results reveal that GXM10-Ac_3 adopts an extended structure, with flexible Xyl/GlcA branches alternating sides along the α-mannan backbone. The *O*-acetyl esters also alternate sides and are grouped in pairs. MD analysis of a twelve M2-repeating unit (RU) polymer, 72 residues, allow us to generalize the GXM10-Ac_3 shape on the PS. We determine that GXM10-Ac_3 structure is on average uniformly represented throughout the polysaccharide. In addition, MD predicts that although the PS can form a helical motif with a pitch of $\sim$2.2 RU, it is short lived. Furthermore, the transient helix is significantly longer than GXM10-Ac_3

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(1.6 RU). Therefore, the weak helical pattern observed by MD is unlikely to be important for most of the tested anti-GXM mAbs. Thus, our data suggest that the structural elements important for Ab recognition are the proximal pair of *O*-acetyl groups and the GlcA placement at defined intervals alternating orientation. Overall, the deduced GXM PS structure provides a foundation for future studies to characterize interactions between cryptococcal GXM PS and host immune factors.

(P133) Establishment of Blood Glycosidase Activities and their Excursions in Sepsis

Kathryn Smith[1], Benjamin S. Haslund-Gourley[1], Peter V. Aziz[1], Douglas M. Heithoff[2], Damien Restagno[1], Jeffrey C. Fried[3], Mai-Britt Ilse[4], Hannah Bäeumges[4], Michael J. Mahan[2], Torben Lübke[4], Jamey D. Marth[1]

[1]Sanford Burnham Prebys Medical Discovery Institute, Infectious and Inflammatory Diseases Center, La Jolla, California 92037, [2]Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, California 93106, [3]Department of Pulmonary and Critical Care Medicine, Cottage Hospital of Santa Barbara, Santa Barbara, California 93105, [4]Department of Chemistry, Biochemistry, Bielefeld University, D-33615, Germany,

Glycosidases are hydrolytic enzymes studied principally in the context of intracellular catabolism within the lysosome. Therefore, glycosidase activities are classically measured in experimentally acidified assay conditions reflecting their low pH optima. However, glycosidases are also present the bloodstream where they may retain sufficient activity to participate in functions including the regulation of glycoprotein half-lives, proteostasis, and disease pathogenesis. We have herein established at physiological pH 7.4 in blood plasma and sera the normal ranges of four major glycosidase activities essential for blood glycoprotein remodeling in healthy mice and humans. These activities included β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase, and α-fucosidase. We have identified their origins to include the mammalian genes *Glb1*, *HexB*, *Man2a1*, and *FucA1*. In experimental sepsis, excursions of glycosidase activities occurred with differences in host responses to discrete bacterial pathogens. Analyses of mannosidase activity in non-septic humans suggests the possibility of a population bearing half the level of activity compared to the remaining population samples analyzed. Further studies are underway to investigate this possibility and its potential impact on physiology. Among glycosidase excursions in human sepsis, the elevation of β-galactosidase activity was a prognostic indicator of increased likelihood of patient death.

(P134) Cell-dependent variable glycosylation of envelope glycoprotein (Env) impacts HIV-1 infectivity and neutralization

Qing Wei[1], Qing Wei[1], Zhengrong Yang[1], Barbora Knoppova[1], Audra Hargett[1], Stacy Hall[1], Rhubell Brown[1], Zina Moldoveanu[1], Janet Yother[1], Milan Raska[2], Todd J. Green[1], Matthew B. Renfrow[1,†], Jan Novak[1,†]

[1]University of Alabama at Birmingham, Birmingham, AL, USA, [2]Department of Immunology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc, Czech Republic,

[†]Co-senior authors

Env, a trimer of gp120 and gp41, is the sole surface antigen of HIV-1. N-glycans represent >50% of gp120 molecular mass, forming a "glycan shield". These glycans impact many Env functions, including virus-cell interaction (binding to CD4 receptor), immune recognition and evasion (reduced sensitivity to broadly neutralizing antibodies [BnAbs]). The Env glycan shield is variable, including the sites of glycan attachment and glycan composition. We postulate that Env glycan shield can be defined as distinct structural glycan-microdomains that complement function and stability of the protein domains in the Env trimer. To better understand the function of Env glycan-microdomains, we have assessed the effects of cell-specific glycosylation using Env-pseudotyped virus and recombinant trimeric gp120 (rgp120). We used Env from an immune-escape clade B HIV-1 (WEAU-d391; WT) and Env modified by removing 2 glycans that restrict access to the CD4-binding site (CD4bs) (WEAU-d391-N276D-N463D; CD4bs-Mut). Env-pseudotyped HIV-1 and rgp120 were produced in FreeStyle 293-F (FS) and Expi293F (XP) cells. TZM-bl reporter cells were used to determine infectivity of the Env-pseudotyped virus and the sensitivity to the soluble CD4 (sCD4) and BnAbs, such as those specific for the CD4bs (VRC01) or the apex of the Env trimer (PG16). Analyses of glycosylation of rgp120 included monosaccharide compositional analysis and glycan profiling; site-specific glycosylation was analyzed by high-resolution mass spectrometry. Structural modeling was used to interpret the glycomics and functional data. Infectivity of XP-produced WT virus was reduced compared to virus produced in FS cells. CD4bs-Mut vs. WT virus produced in FS cells was 3-fold more sensitive to inhibition by sCD4. However, CD4bs-Mut as well as WT virus produced in XP cells were each less sensitive to sCD4 inhibition. VRC01 neutralized WT virus produced in FS and XP cells similarly and CD4bs-Mut viruses produced in both cell types were $\sim$7-fold more sensitive to VRC01. PG16 effectively neutralized WT virus produced in FS cells whereas the CD4bs-Mut viruses were $\sim$4-fold less sensitive. Conversely, WT and CD4bs-Mut viruses produced in XP cells were >8-fold less sensitive to PG16 compared to the WT virus produced in FS cells. Control BnAb (PGT122) neutralized all viruses similarly. rgp120 proteins from XP had a higher content of mannose and high-mannose glycans than those from FS cells. Site-specific glycosylation of rgp120 from XP vs. FS cells showed elevated content of high-mannose vs. complex glycans

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attached at N160, the key glycan for PG16 binding. Structural data show PG16 accommodating high-mannose glycans. Our modeling indicated that complex glycans at N160 could alter PG16 binding, suggesting other structural features may impact the apex glycans. In summary, our results revealed that cell-dependent variable Env glycosylation impacts HIV-1 infectivity in a manner consistent with distinct structural glycan-microdomains.

(P135) IL-6-Induced Potentiation of Platelet Activity and Glycosylation in Venous Thromboembolism

Michelle Brenner[1], Karin Hoffmeister[2], Brian Branchford[1]

[1]*Pediatric Hematology/Oncology/Bone Marrow Transplant, Children's Wisconsin and Medical College of Wisconsin,* [2]*Translational Glycomics Center, Versiti Blood Research Institute,*

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), affects nearly 1 of every 100 hospitalized children in the US each year. VTE is associated with both increased mortality (up to 2%) and morbidity, such as increased healthcare costs due to longer stays, loss of vascular access, chronic thromboembolic pulmonary hypertension, and post-thrombotic syndrome.The specific role of activated platelets in VTE formation has yet to be fully elucidated. It is well known that post-translational modification (such as glycosylation) of platelet adhesion receptors affects platelet activity, localization, and adhesion. Infection can induce desialylation of platelets and lead to the formation of an N-acetylgalactosamine terminus with a greatly enhanced affinity for hepatic Ashwell-Morell receptor (AMR) binding and subsequent thrombocytopenia. In VTE, there is a marked inflammatory response as evidenced by increased inflammatory markers such as C-reactive protein, IL-6, IL-8, and TNF-α. Platelets, like all cells, express the receptor gp130 that can be stimulated by soluble IL-6 receptor to initiate the trans-signaling pathway of IL-6. Platelets were obtained from healthy human donors and stimulated with various concentrations of human recombinant IL-6 ranging from doses comparable to patients receiving IL-6 immunotherapy up to meningococcal septic shock. IL-6 alone does not stimulate platelet aggregation or activation as assessed by p-selectin expression. Addition of lower dose IL-6 leads to inhibition of platelet aggregation and activation via p-selectin expression in response to collagen and TRAP (thrombin receptor activated peptide), however there is no difference with addition of high dose IL-6 as seen in sepsis.Ongoing work and next steps involve analyzing how IL-6 impacts the changes in platelet glycosylation via lectin arrays and flow cytometry (neuraminidase 1, PNA, RCA-1) to identify changes of glycosylation of platelet adhesion proteins and the pathways that demonstrate these differential platelet responses to activation. As such, these glycosylation changes may be a common pattern during thromboembolic events, which may lead to prediction of VTE, earlier identification of VTE, and provide future treatment/prophylaxis options for VTE.

(P136) Galectin-3 controls Helicobacter pylori-induced apoptosis in gastric epithelial cells

Yu-Hsien Hung[1], Fang-Yen Li[2], Fu-Tong Liu[3]

[1]*Department of Medical Research and Development, Chang Gung Memorial Hospital, Linkou, Taiwan,* [2]*Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan,* [3]*Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan,*

Helicobacter pylori (*H. pylori*) infection is a major risk factor for gastric diseases, including gastric ulcer and cancer, and its interaction with host cells remains a subject of intense investigation. We explored the previously understudied role of galectin-3, a β-galactoside-binding lectin, in *H. pylori*-induced apoptosis and its potential link to endo-lysosomal damage. Using AGS cells, a gastric epithelial cell line, we observed that *H. pylori* induces apoptosis, and notably, this process is positively regulated by galectin-3, as determined by comparing galectin-3 knockout cells with wild-type cells. This led us to explore the regulatory mechanisms underlying this phenomenon. We detected the formation of galectin-3 aggregates (puncta) that colocalized with lysosomes in cells infected by *H. pylori*, suggesting their association with pathogen-induced lysosomal damage. Further analysis revealed a positive correlation between galectin-3 puncta formation and *H. pylori*-induced apoptosis. While *H. pylori* vacuolating cytotoxin A (*VacA*) is responsible for apoptosis induction, it does not influence galectin-3 puncta formation, indicating that galectin-3 aggregation is not a prerequisite for *H. pylori*-induced apoptosis. Additionally, although galectin-3 puncta colocalize with the autophagy marker LC3, our findings suggest that galectin-3 does not play a role in autophagic induction, as the amount of LC3 puncta was not affected by the deletion of galectin-3. However, in the absence of Atg5, a key autophagy factor, *H. pylori*-induced apoptosis increased, indicating that autophagic initiation influences apoptosis. Superresolution structural illumination microscopy revealed that *H. pylori*-induced galectin-3 puncta and galectin-8 puncta reside in distinct autophagosome subdomains. In summary, our research emphasizes the significant regulatory function of galectin-3 in triggering apoptosis as a response to *H. pylori* infection. By comprehending the exact molecular pathways that involve galectin-3 in the context of *H. pylori* infection, we may potentially identify novel therapies for gastric illnesses caused by this pathogen.

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(P137) Structure and Function Studies of IgG Autoantibodies Specific for Aberrantly O-glycosylated IgA1 in IgA Nephropathy

Todd Green[1], Jordan Lingo[1], Barbora Knoppova[1], Shihong Qiu[1], Qiao Shang[1], Stacy D. Hall[1], Zhi-Qiang Huang[1], Hitoshi Suzuki[2], Jan Novak[1]

[1]*Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA,* [2]*Department of Nephrology, Juntendo University Urayasu Hospital, Chiba, Japan,*

IgA nephropathy (IgAN), the most common primary glomerulonephritis worldwide, is a major cause of kidney failure. The current pathogenesis model of IgAN is based on the findings that most IgAN patients exhibit immunological defects resulting in elevated levels of aberrantly glycosylated IgA1, with some O-glycans being galactose-deficient (galactose-deficient IgA1; Gd-IgA1), and production of Gd-IgA1-specific IgG autoantibodies (autoAbs). Consequently, pathogenic immune complexes (ICs) are formed in the circulation, some of which deposit in the glomeruli and induce kidney injury. We have shown that a recombinant IgG (rIgG) autoAb, derived from an IgAN patient, bound Gd-IgA1 to form ICs and that such ICs induced mesangioproliferative glomerular injury in a passive mouse model of IgAN. To develop a better understanding of the interactions between Gd-IgA1 and the corresponding autoAbs, we initiated studies of rIgG autoAbs using site-directed mutagenesis approaches, structural studies, and functional analyses.rIgG autoAbs were expressed in Expi293F cells and purified by affinity chromatography. Fabs of the rIgGs were crystallized and studied by X-ray crystallographic methods. Site directed mutagenesis was used to generate a panel of IgG variants with specific amino-acid replacements. Binding of rIgG autoAbs to Gd-IgA1 was assessed by ELISA.We determined structures of Fabs from a patient-derived rIgG autoAb and a healthy control-derived IgG. The former exhibited high- and the latter low-level binding to Gd-IgA1 in ELISA. We used the atomic models to infer mutations of the rIgGs that might affect Gd-IgA1 binding. ELISA-based binding assays of rIgG and variant autoAbs showed that the parent IgAN-derived rIgG bound to Gd-IgA1 as expected, whereas variant rIgGs showed diminished or no binding. We identified amino acids in CDR1, CDR3, and framework regions of the rIgG autoAb that were essential for binding to Gd-IgA1. Structures of variant rIgG autoAbs harboring point mutations that eliminated or diminished Gd-IgA1 binding revealed the Fabs maintained the overall structures with minimal differences, suggesting that the parent side chains at these point mutation sites were likely integral to Gd-IgA1 binding. To complement these findings, we reverse-engineered a healthy control-derived IgG to enhance the binding to Gd-IgA1. Structures of those Fabs indicated that an increased flexibility of the light-chain framework region 3 is likely related to the enhanced Gd-IgA1 binding.We have used a workflow of structural and functional techniques to identify amino acids and regions that are essential for the recognition of Gd-IgA1 by IgG autoAbs. These studies are a first step in mapping the residues of IgG autoAbs that are important for the formation of pathogenic ICs. A better understanding of this interaction could lead to therapeutic approaches aimed to block IgG autoAb-Gd-IgA1 interactions.

(P138) The bacterial effector NleA is modified by host-mediated O-linked glycosylation

Lindsay Burns[1], Natalia Giannakopoulou[1], Alexandra Kazanova[1], Lei Zhu[1], Yong Zhong Xu[2], Rufaida H. Khan[3,4], Sadjia Bekal[3,5], Erwin Schurr[2,6], Martin Schmeing[6,7], Samantha Gruenheid[1]

[1]*McGill Research Centre on Complex Traits and Department of Microbiology and Immunology, McGill University, Montréal, QC, Canada,* [2]*Program in Infectious Diseases and Global Health, The Research Institute of the McGill University Health Centre and McGill International TB Centre, Department of Medicine, Faculty of Medicine, McGill University, Montréal, QC, Canada,* [3]*Laboratoire de santé publique du Québec, Institut national de santé publique du Québec, Sainte-Anne-de-Bellevue, QC, Canada,* [4]*Department of Food Science and Agricultural Chemistry, McGill University, Sainte-Anne-de-Bellevue, QC, Canada,* [5]*Département de Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montréal, QC, Canada,* [6]*Department of Biochemistry, Faculty of Medicine, McGill University, Montréal, QC, Canada,* [7]*Centre de Recherche en Biologie Structurale, McGill University, Montréal, QC, Canada*

Escherichia coli is a ubiquitous member of the human intestinal microbiota. Although commensal *E. coli* and its human host generally coexist with mutual benefits in digestion and immunity, diarrheagenic *E. coli* are gastrointestinal pathogens that have acquired virulence factors that permit them to cause a wide range of enteric or diarrheal diseases in healthy individuals, resulting in more than 500 million illnesses and nearly 200,000 deaths each year. Enteropathogenic *E. coli* (EPEC) is a diarrheagenic strain estimated to cause 30–40% of all infantile diarrhea in developing countries. EPEC utilizes an arsenal of proteins to infect their human hosts, termed virulence factors, which generally alter host cell biology to favour survival and replication of the pathogen. One of the most important virulence factors, injected by EPEC upon adherence, is NleA. *In vivo* experiments have shown that NleA is necessary for pathogenesis, however, the mechanism by which NleA contributes to disease remains largely unknown. We have determined that following translocation into host cells, a serine- and threonine-rich region of NleA is modified by host-mediated mucin-type O-linked glycosylation. Importantly, this region was absent in several clinical isolates. When expressed in *C. rodentium*, a non-modifiable variant of NleA was indistinguishable from wildtype NleA in an acute mortality model but conferred a modest increase in persistence over the course of infection in mixed infections in C57BL/6J mice. This is the first known example of a bacterial effector being modified by host-mediated O-linked glycosylation. Our data also suggests that this

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modification may confer a selective disadvantage to the bacteria during *in vivo* infection. Preliminary *in vitro* work indicates that the presence of the modification may be beneficial to NleA's stability within the host cell. Altogether, this study contributes to a growing understanding of the range of interactions possible between bacterial effector proteins and host cells. Further investigation into the mechanism of NleA function, how it is modified in host cells, and how this modification modulates its function promises to provide new insights into host-microbe interactions.

(P139) Layilin as a Modulator of Platelet Activation and Thrombinflammation

Rebecca Mellema[1], Kimberly A. Queisser[1], Aaron C. Petrey[1,2,3]

[1]*University of Utah Molecular Medicine Program, Salt Lake City, Utah, 84112,* [2]*Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah, 84132,* [3]*Division of Gastroenterology, Department of Internal Medicine, University of Utah, Salt Lake City, Utah, USA,*

Platelets are critical modulators of inflammatory responses, being able to interact with the ever-altering Extracellular Matrix (ECM) during inflammatory and immune responses. One ECM component, the glycosaminoglycan hyaluronan (HA), forms unique pro-inflammatory cable-like structures that recruit incoming leukocytes and platelets during disease or injury. Platelets play a key role during HA-metabolism, binding and cleaving HA with surface-expressed hyluronidase-2 (HYAL-2). Our lab has previously shown that dysregulation of this platelet-mediate HA metabolism contributes to disease severity in inflammatory bowel disease (IBD) through advanced leukocyte recruitment and formation of microthrombi. Platelet HYAL-2 cannot remodel HA without a co-receptor to recognize HA during an immune response. We determined that the most prevalent HA receptor was the C-type lectin receptor layilin, rather than CD44. We also show that layilin is the primary HA receptor on platelets. Therefore, we sought to determine the role of layilin in platelet function. Using transmission aggregometry, we exposed healthy human platelets to HA and LAYN-specific antibody, resulting in significant reduction of platelet aggregation by 60% and 90%, respectively. We examined platelet activation in the presence of the layilin antibody, resulting in a 50% decrease in activation measured by surface p-selectin and active $\alpha_{IIb}\beta_3$. As inside-out integrin $\alpha_{IIb}\beta_3$ activation is crucial to platelet aggregation and activation through fibrinogen binding, we examined $\alpha_{IIb}\beta_3$ function through platelet spreading assays. We found that human platelets, when incubated with layilin-binding antibody, had a loss in platelet spreading phonotype on three ECM components: HA, collagen, and fibrinogen. This is characteristic of loss in integrin $\alpha_{IIb}\beta_3$ function. We found that murine LAYN$^{-/-}$ platelets are hypersensitive, displaying significantly increased activation of integrin $\alpha_{IIb}\beta_3$ compared to WT platelets. We hypothesize that layilin mediates integrin $\alpha_{IIb}\beta_3$ inside-out signaling. As layilin regulates the RhoA/ROCK pathway in epithelial cells, we sought to quantify similar small GTPases upstream of $\alpha_{IIb}\beta_3$ activation associated with cytoskeletal remodeling. Through GTPase pulldown assays, we found that, compared to WT platelets, resting LAYN$^{-/-}$ murine platelets contained ~1.5 times more activated Rac1-GTP, but lower expression levels of the Rac1 inhibitor, p-merlin, upon stimulation. In disease, we found a reduction in layilin protein expression in platelets isolated from inflammatory bowel disease (IBD) patients; platelets isolated from DSS colitis murine platelets replicated these findings with a 60% decrease in protein expression. This coincides with the hyperactive state and loss of HA remodeling in IBD patient platelets. We propose a novel regulatory mechanism wherein platelet layilin mediates $\alpha_{IIb}\beta_3$ through the RAC1/merlin/PAK axis, and loss of this mechanism dysregulates platelet activation in disease.

(P140) Improved glycosynthase enzymes for IgG-specific glycoengineering

Tala Azzam[1], Jonathan J. Du[1,2], Eric J. Sundberg[1]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, 30322,* [2]*Present Address: Sydney Pharmacy School, Faculty of Medicine and Health, The University of Sydney, Camperdown, NSW Australia 2050,*

IgGs have a conserved N-linked glycan attached to residue Asn297 of the homodimeric Fc region which modulates antibody effector functions, a critical aspect of the immune response, by altering binding affinities of Fc receptors and complement. Accordingly, IgG Fc glycosylation has emerged as an important modulatory feature of monoclonal antibody (mAb) therapeutics. However, the production of mAbs often results in a heterogeneous mix of glycoforms, highlighting the importance of chemoenzymatic glycoengineering to achieve homogeneity and specificity.

EndoS2, an IgG-specific endo-β-N-acetylglucosaminidase (ENGase), is one useful enzyme for such remodeling. It acts on complex, high-mannose, and hybrid-type glycans and can be made into a glycosynthase by mutating one of the residues in the catalytic triad, D184, to methionine. Although EndoS2D184M can transfer an oxazoline-linked glycan onto IgGs, it still retains some hydrolytic activity. Thus, we sought to improve the glycosynthase function of EndoS2D184M.

We introduced novel mutations to EndoS2D184M in a non-catalytic residue in the active site, I185. Applying our Specificity of Enzymatic Activity and Kinetics (SEAK) method, we compared the kinetics of the transglycosylation reactions of the mutants using deglycosylated Rituximab and oxazoline linked sialylated bi-antennary complex type (SCT) N-glycan (ox-S2G2) as substrates. Substrate-dependent improved transglycosylation and reduced residual hydrolysis suggests that subtle conformational remodeling of the enzyme's active site may affect catalysis.

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(P141) Poly-LacNAc marker of unique cell types: patients with Multisystem Inflammatory Syndrome in Children have a unique glycan signature compared to patients with acute COVID-19 and convalescent COVID-19 patients

Rebecca Hale[1], Dominique Morais[2], Ryan P. Jajosky[2,4], Megan Elkins[1], Connie M. Arthur[2,3], Janet Chou[1], Sean Stowell[2,3]

[1]*Division of Immunology, Boston Children's Hospital, Harvard Medical School, Boston, MA,* [2]*Joint Program in Transfusion Medicine, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA,* [3]*Harvard Glycomics Center, Harvard Medical School, Boston, MA,* [4]*Biconcavity Inc., Lilburn, GA,*

Glycans are key regulators of immune cell function. They play a critical role in T cell interaction and function, modulating activation, cell signaling and apoptosis. Despite the growing literature highlighting the important role of glycosylation in T cell function, glycan analysis is not integrated into clinical immune phenotyping. We hypothesized that integrating the glycan signature with current methods of immune phenotyping, namely cluster of differentiation (CD) markers, would provide unique information regarding immune status and identify distinct immune cell sub-types which appeared homogenous based on commonly employed CD and similar markers used to currently differentiate distinct immune populations alone. To test this hypothesis, we utilized peripheral blood mononuclear cells (PBMCs) from a biobank at Boston Children's Hospital from hospitalized pediatric patients with Multisystem Inflammatory Syndrome in Children (MIS-C) and acute coronavirus disease 19 (COVID-19) as well as convalescent COVID-19 patients. MIS-C is a post-infectious complication of COVID-19 defined by hyperinflammation and multiorgan involvement. This allowed us to compare the glycan signature of T cells during acute infection, post-infectious hyperinflammation and after resolution of infection. We identified a unique glycan signature for patients with MIS-C when compared to patients with acute and convalescent COVID. Patients with MIS-C had a statistically significant increase in poly-N-acetyl-lactosamine (poly-LacNAc) expression on several T-cell subsets, all known to play an important role in MISC pathogenesis, including CD8+ effector T cells, CD8+ exhausted and senescent T cells (CD8+CD57+PD1+) and CD4+ terminally differentiated (TEMRA) cells (CD4+CCR7-CD45RA+). Similar poly-LacNAc expression was found on patients' CD4+ T regulatory T cells and T follicular helper cells in all patient populations tested, therefore it was not increased in all T cell subsets implicated in the pathogenesis of MIS-C. In summary, using a robust flow cytometry panel, we mapped the T cell glycan signature of a healthy cohort and compared it to hospitalized patients with acute COVID and MIS-C. We found a unique glycan signature for pediatric patients with MIS-C compared to acute and convalescent COVID. We found increased poly-LacNAc on terminally differentiated and exhausted senescent CD4+ and CD8+ T cells respectively, as well on CD8+ effector T cells. Based on only the glycan signature, specifically poly-LacNAc expression on CD4+ TEMRA cells, we were able to identify a patient as severe MIS-C, later corroborated with clinical data. In doing so, the glycan signature provided unique insight into specific features of distinct immune populations that associate with distinct disease entities not otherwise observed when utilizing standard flow cytometry approaches.

(P142) Defining N-glycosylation of immune cell infiltrate in prostate cancer tissues

Kameisha Rashford[1], Lyndsay Young[1], Caroline Kittrell[1], Richard Drake[1]

[1]*Medical University of South Carolina,*

Prostate cancer (PCA) is the second most frequently diagnosed cancer in men worldwide and one of the least responsive to immunotherapy. The effectiveness of immunotherapy is hindered by an immunosuppressive "cold" tumor microenvironment (TME) that supports tumor growth and prevents the ability of the immune system to identify and eliminate cancerous cells. Prostate cancer is often described as a cold tumor with a diverse population of immune infiltrating cells. Using a cohort of 25 human prostate cancer tissues with notable immune cell clusters present, we used a combination of N-glycan imaging mass spectrometry and MALDI-MS immunohistochemistry (MALDI-IHC) workflows to determine immune cell types present and their N-glycan signature. Immune cell cluster sizes in these tissues ranged from 40 – 800 microns in width. The most abundant immune cell types determined by MALDI-IHC were B cells mixed with smaller populations of T cells. A small subset of tissues had robust N-glycan signatures associated with the immune cell clusters, comprised primarily of high mannose and bisecting bi-, tri-, and tetra-antennary compositions. In a majority of tissues evaluated, there were far fewer N-glycan species detected in the immune clusters at lower intensity values relative to the most robust tissues. This would be consistent with a tumor microenvironment deficient in metabolites due to the presence of the tumor, or other immunosuppressive mechanisms. We hypothesize that N-glycans detected by imaging mass spectrometry in tissue immune cell clusters represent tumors with more active immune functional states, and the lack of detection represents immune-suppressed tumors. The combined MALDI-IHC and N-glycan imaging MS analysis of immune cells in the TME could be useful for monitoring new and emerging chemo- and immunotherapies.

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(P143) Discovery of a new sialic acid-binding site of Siglec-7 that frequently occurs among CD33-like Siglecs

Atsushi Yoshimura[1,2], Sayo Morishita[1,2], Mayu Miyashita[1,2], Hinano Komura[1,2], Masaya Hane[1,2,3], Di Wu[1,2,3], Ken Kitajima[1,2,3], Chihiro Sato[1]

[1]*Biosci. Biotech. Center, Nagoya Univ.,* [2]*Grad. Sch. Bioagr. Sci., Nagoya Univ.,* [3]*iGCORE, Nagoya Univ.,*

The sialic acid-binding immunoglobulin-like lectin (Siglec) is a receptor that recognizes Sia residues in glycans and regulates the immune system. Siglec-7 is a human CD33-like siglec, and is localized predominantly on human natural killer (NK) cells and monocytes. Siglec-7 has one Ig-like V-set domain and two Ig-like C2-set domains in the extracellular region that are involved in Sia recognition and binding. The specificity of Siglec-7 is α2,8-diSia or branched α2,6-Sia based on several reports; however, the Sia recognition mechanism is unclear. To understand new insights into the ligand-binding properties of Siglec-7, we carried out *in silico* analysis and site-directed mutagenesis, and found a new sialic acid-binding region (site 2 containing R67), in addition to the well-known primary ligand-binding region (site 1 containing R124). Based on the comparison of the amino acid sequence among Siglecs, we found new essential R residues in site 2, one of which is completely conserved among all identified Siglecs. A mutation analysis of R67, R94 and other amino acid residues in Siglec-7 revealed the disappearance of the Sia binding property, similar to a site 1 mutation (R124A), suggesting that, in addition to site 1, the conserved R residue among Siglecs in site 2 is another functional site.

(P144) Glycan masking of H7 influenza virus HA head domain for epitope-focused immunogen design

Cristina Elisa Martina[1], Iuliia Gilchuk[2], Clara T. Schoeder[3], James E. Crowe, Jr.[2], Jens Meiler[1,3]

[1]*Department of Chemistry and Center for Structural Biology, Vanderbilt University, Nashville, USA,* [2]*Vanderbilt Vaccine Center, Vanderbilt University Medical Center, Nashville, USA,* [3]*Institute for Drug Discovery, Leipzig University Medical School, Leipzig, Germany,*

Influenza virus rapidly mutates and escapes the immune system response. Antibodies targeting the viral surface glycoproteins are unable to recognize the antigen if mutations occurs at the binding interface. In particular, the creation of a glycosylation site and the addition of a sugar chain at the antibody interface can completely abrogate antibody binding. This phenomenon, referred as glycan masking, has been adopted in epitope-focused vaccine design to hide specific epitopes associated to lower therapeutic effects and to better expose sites of vulnerability to the immune system.In this work, we applied the glycan masking technique to design immunogen candidates based on the H7 hemagglutinin head (H7-head) glycoprotein of influenza virus, with a particular focus on the Trimer Interface site II (TI-II) epitope, which is targeted by the protective human monoclonal antibody H7-200. We identified 25 sites on the H7-head, excluding the H7-200 epitope, which are highly prone to glycosylation. Each site is well exposed on the surface of the protein and contains one of the three residues forming the sequon for N-glycosylation NxS/T. We then combined the 25 glycosylation sites in 20 H7-head variants: ten immunogen candidates presented three extra glycosylation sites in addition to the native one (Tri mutants), seven presented five extra glycosylation sites (Penta mutants) and three presented seven extra glycosylation sites (Hepta mutants). The Rosetta Suite, a program for protein modeling and design, was used to model the glycans and to predict changes in antibody binding for nine antigen-antibody pairs with available experimental structure (H7-200, m826, H7-235, FluA-20, H7.5, H7-167, L3A-44, L4A-14 and HNIgGA6). In particular, Rosetta was used to: 1) insert the sequon for N-glycosylation in the structure of H7-head; 2) model the 3-dimensional conformation of the glycan chains; 3) dock the panel of known antibodies at their respective binding site, and 4) predict changes in antibody binding for each antigen-antibody pair. A panel of 16 additional antibodies without known structure was also included in the experimental testing of the H7 head variants. The full panel of 24 antibodies covers the five major antigenic sites of the H7 head domain: TI-II, Trimeric Interface I (TI-I), Site A, Receptor Binding Site (RBS) and Apex. Of the 20 candidates, only ten expressed well as recombinant proteins and all of them bound to H7-200, as predicted by Rosetta. Binding was also retained for the antibodies targeting the Trimer Interface site I (TI-I), while partial or complete loss of binding was shown for antibodies targeting the receptor binding site (RBS), the Apex and the Site A epitopes. Finally, we identified two different mechanisms of binding abrogation mediated by the glycosylations.

(P145) Cryptococcal species capsular and exopolysaccharides are structurally distinct

Maggie Wear[1], Davin Kim[1], Risha Roy[1], Arturo Casadevall[1]

[1]*Johns Hopkins Bloomberg School of Public Health, Baltimore, MD,*

Cryptococcus neoformans sits atop the WHO fungal priority pathogens list. While several factors influence this, the challenge in characterizing and exploiting the polysaccharides *C. neoformans* produces contributes to all of them. Over half a century

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of research has focused on cryptococcal polysaccharides, yet progress has been hindered not just by the complexity of these polymers, but also assumptions about them. For years researchers have assumed that cryptococcal serotype is linked to capsular polysaccharide and the motifs of the predominant polymer glucuronoxylomannan (GXM). However, a search through the original studies shows no such link. In addition, studies examining cryptococcal polysaccharides have conflated shed exopolysaccharide (EPS) with capsular polysaccharide (CPS). Here we show data detailing the differences between EPS and CPS of the cryptococcal species complex. To compare EPS and CPS we have employed four cryptococcal strains, two *C. neoformans* and two *C. gattii*, each strain expresses a single motif of GXM. Utilizing three different polysaccharide quantification techniques (phenol sulfuric acid (PSA) assay, size exclusion chromatography multi-angle light scattering (SEC-MALS) and dry weight) we observe greater yields of CPS per cell than EPS in all four strains. SEC-MALS further shows that while EPS contains three size populations, CPS only contains two. The predominant size population by SEC-MALS varies by species with *C. neoformans* EPS being ∼150 kDa in size while *C gattii* EPS is only ∼41 kDa in size. While smaller in size, the CPS also differs with *C. neoformans* CPS being ∼30 kDa and *C. gattii* CPS being ∼17 kDa. We also examined the protein content (Bradford assay) of these four strains and observed greater amounts of protein in CPS than EPS when normalized to cell count. Polyacrylamide gel electrophoresis (PAGE) with silver staining shows overlapping bands in *C. neoformans* CPS samples and *C. gattii* CPS samples but no overall CPS or EPS overlap was observed. Finally, proton 1D solution state NMR analysis showed the structural reporter group (SRG) region, used to define GXM motifs, to differ between EPS and CPS. While the SRG region of EPS samples shows peak sets attributed to GXM motifs, CPS does not. However, CPS samples contain a conserved doublet peak at 5.18 ppm not observed in the EPS samples. Overall, CPS is smaller in size than EPS, contains more, and likely species specific, proteins, and has a distinct SRG peak set inconsistent with those reported for GXM while EPS is larger in size, contains less protein, and has SRG peak sets consistent with reported GXM motifs. Together these data provide direct evidence that EPS and CPS are distinct species, consistent with the different roles they play during human infection.

(P146) Untangling the web of functional human ligands exploited by Cholera Toxin

Andrew Boucher[1], Akshi Singla[1,2], Michael Lebens[1], Ulf Yrlid[1]

[1]*Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden,* [2]*Department of Medical Chemistry and Cell Biology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden,*

Previous Cholera research has indicated that intoxication occurs solely through GM1 ligands present on intestinal epithelial cells, a product of the extremely high binding affinity detected between GM1 and Cholera Toxin (CT). Despite this, GM1 is virtually undetectable in humans at the main site of CT intoxication, the Small Intestine (SI). Cholera Toxin-B subunit (CTB) instead binds primarily via its alternative so-called non-canonical binding site to SI-Epithelial Cells (SI-ECs), where there is an abundance of lower-affinity ligands expressed.Therefore, we wanted to elucidate the binding site exploited by CT for entry into human SI-EC, to better provide care for those afflicted with Cholera. First, we synthesized simple polymers of solely fucose or galactose residues or a random mix of both attached to a sugar backbone that we incubated with CT prior to treating human donor derived SI organoids (enteroids) to ameliorate intoxication. As an alternative approach, we grew enteroids with inhibitors targeting glycosphingolipids, glycoproteins and specific glycans to establish how CT interacts with the surface ligands available in humans. Additionally, we created single point mutated CTB, depleting the binding capacity of either the canonical, non-canonical or both binding sites to elucidate the glycoconjugate binding site utilized. These methods were then combined to establish the correlation between CT binding and the subsequent effect on intoxication in a human model. We discovered that while GM1 oligosaccharide could inhibit all intoxicative effects of CT, a simple polymer of mixed fucose and galactose could also substantially ameliorate ion efflux at an affordable cost. While inhibition of fucosylation or O-glycosylation results in lower overall CTB binding, enteroids become more sensitive to CT intoxication. This suggests that a potential role of fucosylated glycoconjugate as decoy-like-ligands, protecting the cell from CT and other incoming toxins. Alternatively, the increased sensitivity may be a result of increased cellular expression of Gal/GalNAc terminated glycoconjugates providing more ligands for the canonical site of CT to bind and enter the cell. Combined, the inhibition of fucosylation and O-linked glycosylation results in a substantial increase in canonical site binding ligands, but conversely diminishes the cells sensitivity to CT intoxication. Despite the lack of detectable GM1, the utilization of the canonical site of CT in human intoxication still appears to be required for entry into the cell.Taken together, this shines light on a potential double role where fucosylation may be implicated as both a decoy and a function glycan during CT intoxication.

(P147) Targeting viral essential N-glycans for disease control

Aitor Casas-Sanchez[1], Ryan Siner[1], Jid Purohoo[1]

[1]*Department of Tropical Disease Biology, Liverpool School of Tropical Medicine (UK),*

Most human pathogenic viruses *N*-glycosylate their surface proteins, which are used for host cell recognition and invasion. Using SARS-CoV-2, we showed that by targeting the host protein *N*-glycosylation pathway, either using reverse genetics or

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glycosylation inhibitors, the spread of the infection can be successfully blocked. This is because cells produce fewer virions, and a fraction of these become non-infective to subsequent cells due to defects in spike protein *N*-glycosylation. We also found that infectious virions produced by cells treated with *N*-glycosylation inhibitors are more susceptible to vaccine-derived antibody neutralization. In addition, enzymatic deglycosylation of SARS-CoV-2 virions led to the complete ablation of cell invasion, again evidencing the existence of essential surface *N*-glycans. We have now extrapolated these observations into other flavivirus infection models (e.g., Zika virus, West Nile Virus). Enzymatic removal of the envelope protein N154 *N*-glycan from either mammalian- or mosquito-derived virions with glycosidases recombinantly expressed in *E. coli,* leads to the complete loss of infection in both mosquito and mammalian cells. We are currently experimenting with the specific targeting and faster cleavage of these viral essential glycans by using multimodal recombinant proteins consisting of glycosidases fused to immunoglobulin (scFv) domains. The genes encoding these proteins have the potential to be delivered into the mosquito vectors of these viruses via paratransgenesis.

(P148) Microbial mannnosylinositol phospholipids: Chemical synthesis and biological functions

Akifumi Ito[1], Yohei Arai[1], Kana Okubo[1], Etsuko Nabika[1], Takanori Matsumaru[1], Yukari Fujimoto[1]

[1]*Department of Chemistry, Faculty of Science and Technology, Keio University,*

Microorganisms have characteristic inositol phospholipids such as mannnosylinositol phospholipids, which includes phosphatidylinositol mannosides (PIMs) from *Mycobacterium tuberculosis* and mannosylinositol phosphorylceramides (MIPCs) from *Candida albicans*. They contain *myo*-inositol with mono- or oligo-mannose, in common. PIMs have diacylglycerol moiety as a lipid part, while MIPCs have characteristic ceramide moiety, with various fatty acyl groups in the lipid part. In order to explore the immunological activities of inositol phospholipids, we have developed the methods of chemical synthesis for constructing the compound library of inositol phospholipids. The key synthetic strategy for the inositol phospholipid synthesis includes regio-selective phosphorylation of *myo*-inositol and allyl-type protecting group strategy.[1)] The methods enabled us to synthesize the inositol phospholipid derivatives having almost any kinds of combination of substitution on the *myo*-inositol and lipid parts.

Among various PIMs compounds, we synthesized Ac_1PIM_1, which has mono-acyl group and mono-mannosylated phosphatidylinositol. Selective mannnosylation and phosphorylation with BINOL-phsophoseleno reagent were applied for the synthesis, and we then succeeded in the first synthesis of Ac_1PIM_1.[2)] As for the MIPCs, we applied a different combination of the protecting groups for the mannnosylation of the inositol for more efficient preparation of the intermediates. We also used azirizine ring opening reaction for ceramide introduction. Using these strategy, we newly developed a synthetic strategy for MIPCs, which enables to introduce various lipid moieties. We thus accomplished the total synthesis of MIPCs with several combination of the fatty acids. Having these mannosylinositol phospholipids in hand, we firstly analyzed Ac_1PIM_1 and the related compounds, regarding the activation of innate immune receptors and glycolipid antigen presentation.[2)] We then analyzed the immunobiological activities of MIPCs. Based on the chemically synthesized compounds, we contentiously evaluate the immunomodulatory functions of the ionositol phospholipids.

(P149) N-acetylglucosamine inhibits inflammation and neurodegeneration markers in multiple sclerosis: a mechanistic trial

Michael Sy[1], Barbara L. Newton[1], Judy Pawling[2], Ken Hayama[1], Andres Cordon[1], Zhaoxia Yu[3], Jens Kuhle[4], James W. Dennis[2,5], Alexander U. Brandt[1], Michael Demetriou[1,6,*]

[1]*Department of Neurology, University of California Irvine, 208 Sprague Hall, Mail Code 4032, Irvine, CA 92697, USA,* [2]*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave, Toronto, ON M5G 1X5, Canada,* [3]*Department of Statistics, Donald Bren School of Information and Computer Sciences, University of California Irvine, Bren Hall 2019, Irvine, CA 92697, USA,* [4]*Department of Neurology, University Hospital Basel, Mittlere Strasse 83, 4056 Basel, Switzerland,* [5]*Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada,* [6]*Department of Microbiology and Molecular Genetics, University of California Irvine, Irvine, CA 92697, USA,*

In the demyelinating disease multiple sclerosis (MS), chronic-active brain inflammation, remyelination failure and neurodegeneration remain major issues despite immunotherapy. While B cell depletion and blockade/sequestration of T and B cells potently reduces episodic relapses, they act peripherally to allow persistence of chronic-active brain inflammation and progressive neurological dysfunction. N-acetylglucosamine (GlcNAc) is a brain penetrant triple modulator of inflammation, myelination and neurodegeneration. GlcNAc is a key metabolite regulating Asn (N)-linked-glycans, which interact with galectins to co-regulate the clustering/signaling/endocytosis of multiple glycoproteins simultaneously. In mice, GlcNAc readily crosses the blood brain barrier (BBB) to raise N-glycan branching, suppress inflammatory demyelination by T and B cells and trigger stem/progenitor cell mediated myelin repair to limit neuro-axonal damage. MS clinical severity, demyelination lesion size

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and neurodegeneration inversely associate with a marker of endogenous GlcNAc, while in healthy humans age-associated increases in endogenous GlcNAc promote T cell senescence. Here we report a mechanistic open-label trial assessing oral GlcNAc at 6g (n=18) and 12g daily for four weeks in MS patients not in relapse and on the immunomodulator glatiramer acetate. Prior to GlcNAc therapy, high serum levels of the inflammatory cytokines IFNg, IL-17 and IL-6 associated with reduced baseline levels of a marker of endogenous serum GlcNAc. Oral GlcNAc therapy was safe, raised serum levels and modulated N-glycan branching in lymphocytes. Glatiramer acetate reduces TH1, TH17 and B cell activity, yet the addition of oral GlcNAc dose-dependently lowered serum IFNg, IL-17 and IL-6. Oral GlcANc also dose-dependently reduced serum levels of the anti-inflammatory cytokine IL-10, which is elevated in the brain of MS patients. As glatiramer acetate acts peripherally, the reduction in inflammatory cytokines and IL-10 by oral GlcNAc may arise from crossing the BBB to target chronic-active CNS inflammation. Consistent with this, oral GlcNAc dose-dependently reduced serum neurofilament light chain, a specific marker of brain inflammation/neurodegeneration. Moreover, 30% of treated patients displayed confirmed improvement in neurological disability; suggesting reduced brain inflammation and/or re-myelination. Blinded clinical trials are warranted to validate GlcNAc's potential to control residual brain inflammation, myelin repair and neurodegeneration in MS.

(P150) Molecular modeling and machine learning models to identify FmlH lectin-binding small molecules for the prevention of Escherichia Coli-induced urinary tract infections

Priyanka Samanta[1], Robert J. Doerksen[1,2]

[1]*Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, University, MS, USA, 38677-1848,* [2]*Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS, USA, 38677-1848,*

Approximately 50% of women are affected by UTIs during their lifetime. Microbial agents such as Gram-positive and Gram-negative bacteria and fungi are the primary agents that cause UTIs, and among them the most common is uropathogenic *Escherichia coli* (UPEC). UPEC expresses fibers known as F9/Fml pili tipped with pilus adhesin protein FmlH that specifically bind to terminal galactoside and galactosaminoside units in glycoproteins in kidney and bladder cells and colonize host tissues. The traditional UTI treatment using antibiotics has led to the rise of various UPEC antibiotic-resistant strains. An alternative therapeutic approach includes anti-adhesion strategy to prevent initial bacterial attachment using competitive tight-binding inhibitors. In this study, we have used computer-aided drug design techniques to identify novel glycomimetics that are predicted to bind strongly to and inhibit the UPEC FmlH. We designed a novel virtual screening workflow that included hybrid fragment-based drug design and molecular docking. Some of the predicted inhibitor fragments had been previously identified as UPEC pili inhibitor fragments, thereby validating our workflow. We generated a database containing ∼180K small molecules which could be utilized to identify new glycomimetics that can be subjected to further biological activity studies. We have used machine-learning-based ADMET predictor models to investigate the toxicity and metabolism profiles of the designed glycomimetics. Following this, we have also performed *in silico* receptor-based and ligand-based scaffold hopping techniques to obtained novel FmlH strong-binding glycomimetics with high chemical synthesizability and improved pharmacokinetic profiles.

(P151) Stochastic Protein Glycosylation is Maintained in Minimal Genome Mycoplasma spp

John Sanford[1], James Daubenspeck[1], Lissa Anderson[2], Jason Needham[3], Kevin Dybvig[1], T. Prescott Atkinson[1]

[1]*University of Alabama at Birmingham, Birmingham, AL,* [2]*National High Magnetic Field Laboratory, Tallahassee, FL,* [3]*University of Maryland, College Park, MD,*

The genus *Mycoplasma* is composed of cell wall-less bacteria that have limited metabolic capabilities due to genome streamlining. Mycoplasmas primarily parasitize animals, maintaining sophisticated immune evasion strategies while stealing most nutrients from their host. In addition to their medical and agricultural health relevance, mycoplasmas have two attractive model organisms for studying minimal life. *Mycoplasma genitalium* (Mgen) is a human urogenital pathobiont that has a 581-kbp genome evolved under the selective pressure of host immunity. In 2016, the J. Craig Venter Institute synthesized and transplanted a near-minimal (543-kbp) genome based on *Mycoplasma mycoides*, containing genes essential for growth and a stable cell cycle under axenic culture into a recipient cell, with the resulting organism called JCVI-syn3A (syn3A).

Protein glycosylation is a ubiquitous process found across all forms of life. Previous work in our group determined a surface protein hexosylation system was active in the murine pathobionts *Mycoplasma pulmonis* and *Mycoplasma arthritidis*, as well as the human pathobiont *Mycoplasma pneumoniae*. This hexosylation process uses disaccharides or larger glycans as the activating sugar instead of the canonical UDP-nucleotide seen in most bacterial glycosylation systems. Furthermore, no sequence motif was found for glycosylation sites with cases of *N*- and *O*-linked glycans, indicating it is a highly generalized and stochastic process. We have never found evidence of a complete glycosylation knockout in mycoplasmas, and the few annotated glycosyltransferases in mycoplasmas appear to be essential.

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A currently unknown glycosyltransferase mediates this stochastic hexosylation system and have hypothesized it is active in both the natural and synthetic genome-streamlined mycoplasmas based on periodic acid staining of surface proteins from both Mgen and syn3A. We isolated membranes and performed high-resolution mass spectrometry on in-solution digested peptides from Mgen and syn3A membranes. Our results indicate this hexosylation system is active in both organisms and appears to be as stochastic and generalized as in other, less genome-streamlined, mycoplasmas.

The presence of this hexosylation system in both organisms, especially the non-pathogenic syn3A, strongly implies that this process is essential for mycoplasmas even without the selective pressure of the host immune system. While a stochastic glycosylation system would increase the antigenic variation of a population of mycoplasmas, it may also protect the organism from its own secreted proteases or play a role in another fundamental process for life. A dearth of glycosylation knockouts in the genus furthermore gives credence to the essentiality of this process, which also may make it an attractive target for novel therapeutics against pathogenic mycoplasmas, downstream of understanding the basic biology of the system.

(P152) Distinct T cell subsets express unique glycan signatures that are largely conserved between mouse and human

Dominique Morais[1], Rebecca Hale[2], Shang-Chuen Wu[1], Hau-Ming Jan[1], Carter Stowell[1], Luke Westhoff[1], Sofia Thompson[1], Chien-Yu Kuo[1], Charlotte Noble[1], Fiona Wallace[1], Connie Arthur[1], Sean Stowell[1,3]

[1]Department of Pathology, Joint Program in Transfusion Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, [2]Division of Immunology, Boston Children's Hospital, Harvard Medical School, Boston, MA, [3]Harvard Glycomics Center, Harvard Medical School, Boston, MA,

Tight regulation of the immune system is key to maintaining the delicate balance between an immune response and tolerance. Among immune regulatory factors, glycans and glycan-binding proteins (GBPs) are increasingly recognized as key modulators that help maintain this homeostasis. However, dynamic changes in the glycocalyx of distinct immune populations that regulate their sensitivity to GBP-mediated regulation remains incompletely understood. Several studies have demonstrated that the glycan composition of the glycocalyx of many immune cells, in particular distinct T cell populations, can be highly dynamic and depends on the activation and overall differentiation state of a given cell. However, due to the complex nature of glycans and the lack of tools to probe the glycocalyx, most studies have been limited to evaluating only a few glycan moieties. Furthermore, as the glycan signatures of distinct species can differ, whether a possible change in glycan signatures that accompany T cell activation and differentiation are preserved across species remains incompletely understood. As a result, we sought to define the glycan signatures of distinct T cells in more detail, including the extent to which these signatures are conserved between mouse and human. To this end, we employed a series of lectin probes capable of distinguishing key glycan motifs previously shown to regulate interactions with multiple host GBPs, including poly-N-acetyllactosamine, terminal β-galactose, branched complex N-glycans, α2,6-sialic acid, and α2,3 sialic acid on distinct T cell populations, including T helper 1, T helper 2, T helper 17 and regulatory T cells. Using this panel, we found dynamic changes in the glycan signature between resting, activated and differentiating CD4+ and CD8+ T cells in both mouse and human. Strikingly, there are few differences between the glycan signatures of activated mouse and human T cells, as well as differentiating T cells. However, we did observe notable differences, including increased expression of complex branched N-glycans and terminal β-galactose on human Th17 cells when compared to mouse. Additionally, β-galactose expression varied between mouse T helper subsets, whereas in humans no difference between T helper subsets was detected. In summary, our data demonstrates that the glycan signatures of T cells and their subsets are dynamically regulated and generally conserved between mouse and human, emphasizing the important role these glycans play in modulating the immune system.

(P153) B4GalT1 dependent glycans control the functionally distinct megakaryocyte-biased multipotent progenitor subsets

Karin M. Hoffmeister[1,4], Alejandro Roisman[1], Leonardo Rivadeneyra[1], Linsey Conroy[2], Natalia Weich[1], Joseph T. Lau[3], Ramon Sun[2]

[1]Translational Glycomics Center, Versiti Blood Research Institute, Milwaukee, WI, USA, [2]Department of Biochemistry & Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, USA, [3]Roswell Park Comprehensive Cancer Center, Department of Molecular and Cellular Biology, Buffalo, NY, [4]Departments of Medicine and Biochemistry, Medical College of Wisconsin, Milwaukee, WI,

Emerging research supports a dynamic model of blood development, wherein hematopoietic stem cells (HSCs) play a pivotal role in lineage specification by generating distinct lineage-biased multipotent progenitors (MPP) subsets, such as myeloid-biased MPP2 and MPP3. These subsets, together with lymphoid-primed MPP4 cells, regulate continuous blood production. Interestingly, under regenerative stress, HSCs temporarily overproduce the typically scarce myeloid-biased MPPs, particularly the megakaryocyte (MK)-primed (CD41+) MPP2 subset, promoting myeloid amplification vital for hematopoietic system reconstruction. Despite advances in understanding blood production mechanisms, the role of cell surface and extracellular

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glycans in lineage specification at the MPP level remains unclear.Our recent findings reveal the glycosyltransferase β-1,4-galactosyltransferase 1 (B4GalT1) is crucial in hematopoietic development. Its absence leads to dysplastic megakaryocytes, impaired thrombopoiesis, and a notable increase in HSCs. Detailed analysis of glycan composition in control and B4GalT1 null (B4-/-) femurs using MALDI-MSI revealed a distinct gradient of capped, complex N-glycans in control femurs, with a higher expression at the distal ends and a tapering complexity towards the shaft. This gradient was absent in B4-/- femurs, which instead showed a surge in immature N-glycans and a decrease in complex structures, indicating B4GalT1's role in generating specific bone marrow N-glycan niches. Additionally, loss of capped N-glycans and aberrant O-glycosylation in B4-/- LT-HSCs mirrored cancer-associated glycan patterns. This led to the expansion of long-term HSCs (LT-HSCs), with B4-/- LT-HSCs showing higher expression of the phenotypic MK marker CD41, suggesting specific MPP2 expansion observed under regenerative stress and increased inflammation despite the absence of pro-inflammatory cues in B4-/- mice.Single-cell RNA-seq analysis of the global transcriptional landscape in B4-/- HSCs revealed an expansion of transcriptional output associated with LT-HSC and MK-primed MPP2 signatures, confirming the phenotypic HSC analysis. Gene set enrichment analysis (GSEA) showed metabolism-associated pathways and cell cycle upregulation, while cellular adhesion and lineage commitment were downregulated explicitly in B4-/- LT-HSC CD41+ cells. GSEA revealed upregulated Wnt and Myc pathways in B4-/- LT-HSC CD41+ cells. Treatment with the Wnt inhibitor XAV939 normalized cell numbers and reduced expansion in stem and progenitor compartments, suggesting B4GalT1-dependent glycans control MPP2 expansion via the Wnt/β-catenin signaling pathway, while other MPP compartments, including MPP4, remain unperturbed. The data highlight the role of glycans in hematopoiesis and the significance of the B4GalT1-Wnt-β-catenin axis in LT-HSC fitness and expansion by regulating the emergency responsive MPP2 compartments.

(P154) Antibody Epitope Expression in the Polysaccharide Capsule of Cryptococcus neoformans Changes as a Function of Chronological and Generational Time

Seth Greengo[1], Maggie P. Wear[1], Arturo Casadevall[1]

[1]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA,

Cryptococcus neoformans is unique among eukaryotic pathogens for its ability to grow a capsule under certain conditions, including mammalian infection. The primary component of the capsule is the polysaccharide glucuronoxylomannan (GXM), the repeat unit of which differs by xylose content and linkage to yield six characterized motifs. The motifs expressed by a given *C. neoformans* strain make up surface epitopes and thereby influence antibody binding. Previous studies show that the serotype D *C. neoformans* strain ATCC 24067 can undergo microevolution following infection and continuous culture, resulting in changes to secreted polysaccharide structure as well as antibody binding to the capsule. Differences in antibody binding to the cryptococcal capsule have also been observed between parent cells and buds, indicating that daughter cells express distinct antibody epitopes. Despite this progress, questions remain about the regulation of GXM motif and epitope expression by *C. neoformans*. To characterize the differences between lab cultured AC-24067 and the parent strain ATCC-24067 we utilized complementary techniques of immunofluorescence (IF) microscopy, solution state NMR, and ELISA. NMR analysis of secreted polysaccharide showed structural differences between AC-24067 and ATCC-24067 beyond that which were previously reported. Since NMR does not currently allow for the analysis of capsular polysaccharide, we utilized IF to further explore the differences between these two strains. We found that while mAb 12A1 bound both strains the same, mAb 13F1 binding resulted in different intensities or patterns of staining. Curiously, a recent glycan array analysis found that there is significant overlap in the GXM motifs that these antibodies bind. More interesting still, when cells are chemically locked into the S or M phase of the cell cycle (with hydroxyurea or nocodazole, respectively) mAb 13F1 binds predominantly to the mother cell rather than the daughter bud. Studies examining mAb binding to the capsule have suggested that the capsules of daughter cells must be kept separate from the mother cells to ensure accurate division. Furthermore, past research in our lab has shown that the capsules of chronologically older cells differ in epitope density compared to younger cells. Our present data suggest that beyond physical separation, daughter capsules are also structurally distinct. Given all these data, it appears that the antibody epitopes expressed by a *C. neoformans* cell can change during its lifespan. These differences in capsular structure and epitope expression throughout the aging process may contribute to immune evasion and facilitate chronic infection. These observations add to our understanding of cryptococcal polysaccharide structure and polysaccharide epitope interaction with anti-GXM antibodies. This work will provide great insight into the biology of *C. neoformans* and may inform the design of antibody-based therapeutics.

(P155) A novel lamprey antibody with broad-range specificity for alpha2-6-linked N-Acetylneuraminic acid on mammalian glycoproteins

Lukas Muerner[1], Suttipong Suttapitugsakul[1], Ea K.C. Tulin[1], Catherine A. Tilton[1], Lauren E. Pepi[1], Maxence Noel[1], Tanya R. McKitrick[1], Stephan von Gunten[2], Richard D. Cummings[1]

[1]Department of Surgery, Beth Israel Deaconess Medical Center, National Center for Functional Glycomics, Harvard Medical School, Boston, MA, 02215, U.S.A, [2]Institute of Pharmacology, University of Bern, 3010 Bern, Switzerland,

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Sialylated glycans are critically involved in various biological functions including host-pathogen interaction, immune regulation, as well as regulation of cell migration. However, studies on cellular sialylation often depend on plant lectins such as *Maackia amurensis* agglutinins (MAL-I and -II) and *Sambucus nigra* agglutinin I (SNA-I) which are known to recognize other determinants of glycans besides sialic acid, most prominently sulfated galactose. In attempts to develop novel reagents that recognize sialic acid, we immunized sea lampreys with human peripheral blood mononuclear cells (PBMCs), and generated variable lymphocyte receptor (VLR) yeast surface display libraries (YSDs). This was followed by enrichment of the YSDs for glycan specific antibodies on glycan arrays, and subsequent cloning and expression of relevant antibodies as VLR-rat Fc chimeras. We then characterized the specificity of potentially novel antibodies using multiple screening strategies. One of the newly discovered VLRs we termed PBMC3-02 was found to recognize N-Acetylneuraminic acid (Neu5Ac) α2-6-linked to either galactose (Gal), N-Acetylgalactosamine (GalNAc), or N-Acetylglucosamine (GlcNAc) on glycan microarrays. Notably, all binding was abolished in presence of the hapten 100 mM Neu5Ac. The specificity of PBMC3-02 for α2-6-linked Neu5Ac on O- and N-glycans was corroborated by glycoprotein microarray and western blot analyses, where pretreatment with neuraminidase caused a loss of binding to mammalian glycoproteins, while PNGase F treatment only resulted in a partial loss of binding. To extend these studies on PBMC3-02 specificity, we generated K562 and HEK-293T CRISPR-Cas9 knock out cell lines for various glycogenes. While knocking out the biosynthetic pathways for extended O-glycans (Δ*C1GALT1* and Δ*C1GALT1C1*) and gangliosides (Δ*UGCG*) did not abolish PBMC3-02 binding, loss of complex N-glycans (Δ*MGAT1*) and α2-6-sialylation of N-glycans (Δ*ST6GAL1*) significantly reduced binding, whereas loss of all sialylation (Δ*CMAS*), resulted in complete abrogation of PBMC3-02 binding in flow cytometry and western blot analyses. Furthermore, immunofluorescence staining of different tissue sections as well as flow cytometric analysis on human leukocytes and mammalian cell lines revealed neuraminidase-sensitive binding toward diverse tissues and cell types. Enrichment of human PBMC peptides using SNA-I or PBMC3-02 followed by glycoproteomic analysis identified two sets of sialylated glycoproteins covering diverse families and biological functions. These results confirm that PBMC3-02 has broad-range specificity to α2-6-linked Neu5Ac on mammalian glycoproteins and represents a new and important tool to study the biological roles of sialoglycans. Ultimately, our findings emphasize the potential of the utilized approach to develop antibodies to study the functional roles of glycans in health and disease, as well as for the potential discovery of novel biomarkers and therapeutic targets.

(P156) Clostridioides difficile Infection

Rachel Stuber[1], Janiece Glover[1], Sarah Dooley[1], Richard Drake[2], Amy Engevik[1], Mindy Engevik[1,3]

[1]Department of Regenerative Medicine & Cell Biology, Medical University of South Carolina (MUSC), Charleston SC, [2]Department of Cell and Molecular Pharmacology and Experimental Therapeutics, MUSC, Charleston SC, [3]Department of Microbiology & Immunology, MUSC, Charleston SC,

Background: The colonic mucus layer is a lamellar structure which protects the gastrointestinal tract and maintains gut homeostasis. The intestinal mucus layer is primarily composed of the glycoprotein MUC2. Humans, but not mice, also secrete MUC5B, although to a significantly lower extent than MUC2. Interestingly, recent work has identified that a non-intestinal mucin, MUC5AC, is expressed in mice during infection with the nematode *Trichuris trichiura* and during dextran sodium sulfate induced chemical colitis. MUC5AC is normally expressed in non-intestinal mucosa such as the airway, stomach, and ocular epithelia, but we speculated that similar to mice, human might upregulate colonic MUC5AC in the setting of intestinal inflammation.

Methods & Results: Using CellGene, a publicly available database that houses over 500 datasets, we evaluated mucin expression in various cell types in the human colon. This platform revealed that within the large intestine of healthy individuals, MUC2 is highly expressed in goblet cells. In contrast, MUC5B had limited expression within the large intestine goblet cells and there was little to no detectable levels of MUC5AC. To test the hypothesis that MUC5AC is elevated during inflammation, we obtained surgical resections and biopsies of ascending colon from 9 healthy individuals and 9 patients with recurrent *Clostridioides difficile* infection. By H&E staining, we observed crypt hyperplasia and infiltration of immune cells in *C. difficile* infected patients, consistent with inflammation. As expected, in healthy individuals MUC2 was the dominant colonic mucin. We did observe some MUC5B found in the crypt base, but MUC5AC was largely absent. In contrast to controls, we saw significant levels of MUC5AC in 7 of the 9 *C. difficile* infected individuals. We also observed that all individuals had elevated MUC5B levels, and this protein was being actively secreted. To assess if the glycan profiles were altered, we performed imaging mass spectrometry. We found that *C. difficile* infected colons had altered branched, mannose, fucosylated, and asialyated glycans compared with controls.

Conclusions: These data demonstrate that human goblet cells can generate MUC5AC in response to bacterial-driven inflammation and that *C. difficile* infection alters both the intestinal mucin and glycan profiles.

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(P157) Ultra-sensitive platelet proteome maps the O-glycosylation landscape and identifies a new form of domain-specific *O*-fucosylation

Callum B. Houlahan[1], Yvonne Kong[2], Bede Johnston[1], Michelle Cielesh[3], The Huong Chau[4], Paul R. Coleman[1], Huilin Hao[5], Robert S. Haltiwanger[5], Morten Thaysen-Andersen[4], Freda H. Passam[1,2,*], Mark Larance[3,*]

[1]The Heart Research Institute and Charles Perkins Centre, University of Sydney, Sydney, NSW, Australia, [2]Central Clinical School, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia, [3]Charles Perkins Centre, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia, [4]School of Natural Sciences, Macquarie University, Macquarie Park, NSW, Australia, [5]Complex Carbohydrate Research Center, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, USA,

*Corresponding author

Platelet activation induces the secretion of proteins that promote platelet aggregation and inflammation. However, detailed analysis of the released platelet proteome is hampered by platelets' tendency to pre-activate during their isolation and a lack of sensitive protocols for low abundance releasate analysis. Here we detail the most sensitive analysis to date of the platelet releasate proteome with the detection of >1,300 proteins. Unbiased scanning for post-translational modifications within releasate proteins highlighted *O*-glycosylation as being a major component. For the first time, we detected high stoichiometry *O*-fucosylation on previously uncharacterised sites including multimerin-1 (MMRN1), a major alpha granule protein that supports platelet adhesion to collagen and is a carrier for platelet factor V. The N-terminal elastin microfibril interface (EMI) domain of MMRN1, a key site for protein-protein interaction, was *O*-fucosylated at a conserved threonine (T216) within a novel consensus sequence. Secretion was inhibited of a MMRN1 T216A mutant that could not be *O*-fucosylated on the EMI domain, supporting a functional role of EMI domain *O*-fucosylation in MMRN1 function. Data from interaction predictions using Alphafold2-Multimer and co-immunopreciptation analysis suggest that a widely expressed fucosyltransferase, FUT11, is responsible for this modification. The EMI domain is present in 18 distinct mammalian proteins that are either secreted, or integral membrane proteins, and produced by a diverse array of tissues such as platelets, neurons and muscle. We have confirmed that the EMI domain of another protein, EMILIN-1 was also *O*-fucosylated in its EMI domain, suggesting the widespread necessity of this new modification for protein function.

(P158) Location, location, location: Position-specific N- and O-glycosylation impacts neutrophil elastase-mediated proteolysis of corticosteroid-binding globulin

Anastasia Chernykh[1], Jodie L. Abrahams[1,2], Oliver C. Grant[3], Lucas Kambanis[4,5], Zeynep Sumer-Bayraktar[1,6], Rebeca Kawahara[1,7], Leo Corcilius[4,5], Richard J. Payne[4,5], Robert J. Woods[3], Morten Thaysen-Andersen[1,7]

[1]School of Natural Sciences, Macquarie University, Sydney, NSW 2109, Australia, [2]Glycosciences Laboratory, Department of Metabolism, Digestion and Reproduction, Imperial College London, London W12 0NN, United Kingdom, [3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, United States of America, [4]School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia, [5]Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Sydney, Sydney, NSW 2006, Australia, [6]Glycometabolic Biochemistry Team, Cluster of Pioneering Research, RIKEN, Wako, Saitama 351-0198, Japan, [7]Institute for Glyco-core Research (iGCORE), Nagoya University, Nagoya, Japan

Corticosteroid-binding globulin (CBG) delivers anti-inflammatory cortisol to inflamed tissues through the proteolytic cleavage of an exposed reactive centre loop (RCL) by neutrophil elastase (NE). We previously demonstrated that RCL-localised Asn347-linked *N*-glycans impact NE proteolysis, but a comprehensive structure-function characterisation of the RCL glycosylation is still required to understand the complex CBG glycobiology. Herein, we firstly performed deep RCL-centric glycoprofiling of serum-derived CBG to fully elucidate the Asn347-glycans and then used molecular dynamics (MD) simulations to confirm their impact on NE proteolysis. Importantly, we also identified a hitherto unknown presence of *O*-glycosylation across four discrete RCL sites (Thr338/Thr342/Thr345/Ser350) of serum CBG. Notably, the Thr345-glycans (di/sialyl T) were positioned proximal to the NE-targeted Val344-Thr345 cleavage site. While no multi-*O*-glycosylated CBG glycoforms were observed, some CBG glycoforms displayed an intriguing *N*-/*O*-glycan co-occurrence on the RCL involving exclusively the Asn347/Thr338 sites. The restricted co-occurrence pattern was supported by MD of a GalNAcT-CBG complex, which indicated incompatible Asn347/Thr345 co-occupancy. Glycoprofiling of recombinant CBG (rCBG) revealed high Thr345-glycosylation, which therefore was used to explore the roles of RCL *O*-glycosylation. Longitudinal NE-centric cleavage experiments demonstrated that both sialo- (disialyl T) and asialo-glycans (T) decorating rCBG Thr345 strongly protect against NE proteolysis. Synthetic RCL *O*-glycopeptides expanded on these findings by showing that Thr345-GalNAc (Tn) and Thr342-Tn confer strong and moderate protection against NE cleavage, respectively. MD substantiated that even minimal Thr345-Tn structures abrogate NE interactions. In conclusion, we report on biologically-relevant and strategically-positioned CBG RCL glycans, which improve our understanding of mechanisms governing cortisol delivery to inflamed tissues.

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(P159) Profound N-glycan remodelling accompanies MHC-II immunopeptide presentation

Hayley Goodson[1], Rebeca Kawahara[1,2], Sayantani Chatterjee[1,3], Gabriel Goncalves[4], Joshua Fehring[4], Anthony W. Purcell[4], Nathan P. Croft[4], Morten Thaysen-Andersen[1,2]

[1]*School of Natural Sciences, Macquarie University, Australia,* [2]*Institute for Glyco-core Research (iGCORE), Nagoya University, Japan,* [3]*Department of Biochemistry & Cell Biology, Boston University Chobanian & Avedisian School of Medicine, USA,* [4]*Department of Biochemistry and Molecular Biology & Biomedicine Discovery Institute, Monash University, Australia,*

Immunopeptidomics, the study of peptide antigens presented on the cell surface by the major histocompatibility complex (MHC), offers insights into how our immune system recognises self/non-self in health and disease. We recently discovered that hyper-processed (remodelled) *N*-glycans are dominant features decorating viral spike immunopeptides presented via MHC-class II (MHC-II) molecules by dendritic cells pulsed with SARS-CoV-2 spike protein, but it remains unknown if endogenous immunopeptides also undergo *N*-glycan remodelling. Taking a multi-omics approach, we here interrogate published MHC-II immunopeptidomics datasets of cultured monocyte-like (THP-1) and breast cancer-derived (MDA-MB-231) cell lines for overlooked *N*-glycosylated peptide antigens, which we compare to their source proteins in the cellular glycoproteome using proteomics and *N*-glycomics data from matching cell lines. Hyper-processed chitobiose core and paucimannosidic *N*-glycans alongside under-processed oligomannosidic *N*-glycans were found to prevalently modify MHC-II-bound immunopeptides isolated from both THP-1 and MDA-MB-231, while complex/hybrid-type *N*-glycans were (near-)absent in the immunopeptidome as supported further by new *N*-glycomics data generated from isolated MHC-II-bound peptides derived from MDA-MB-231 cells. Contrastingly, the cellular proteomics and *N*-glycomics data from both cell lines revealed conventional *N*-glycosylation rich in complex/hybrid-type *N*-glycans, which, together with the identification of key lysosomal glycosidases, suggest that MHC-II peptide antigen processing is accompanied by extensive *N*-glycan trimming. *N*-glycan remodelling appeared particularly dramatic for cell surface-located glycoproteins while less remodelling was observed for lysosomal-resident glycoproteins. Collectively, our findings indicate that both under- and hyper-processed *N*-glycans are prevalent features of endogenous MHC-II immunopeptides, an observation that demands further investigation to enable a better molecular-level understanding of immune surveillance.

(P160) Characterizing the glycan-binding properties of gonococcal TonB-dependent transporters

Xiaofan Chen[1], Evgeny A. Semchenko[1], Christopher J. Day[1], Lauren E. Hartley-Tassell[1], Cynthia N. Cornelissen[2], Julie L. Stoudenmire[2], Nicholas Noinaj[3,4,5], Stavros A. Maurakis[2], Kate L. Seib[1]

[1]*Institute for Glycomics, Griffith University, Gold Coast Campus, QLD, Australia,* [2]*Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia, USA,* [3]*Markey Center for Structural Biology, Department of Biological Science, Purdue University, West Lafayette, Indiana, USA,* [4]*Purdue Institute of Inflammation, Immunology and Infectious Disease, Purdue University, West Lafayette, Indiana, USA,* [5]*Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA,*

Neisseria gonorrhoeae, a Gram-negative diplococci bacteria, is the causative agent of gonorrhoea, which is one of the most common sexually transmitted infection globally. Due to the spread of antimicrobial resistance isolates and the lack of a vaccine, gonorrhoea has become an intractable problem. Glycans on the surface of host cells are commonly targeted by pathogens, including *N. gonorrhoeae*. Our lab showed that *N. gonorrhoeae* bind to several glycans with different structural and functional classes, especially glycosaminoglycans and mannosyl glycans. Seventeen potential mannose-binding proteins of *N. gonorrhoeae* were isolated and identified, including the TonB-dependent metal transporters TbpA, TdfJ and TdfH. The principal focus of my research is to characterize the interaction between these gonococcal proteins and glycans and evaluate their potential as targets for new drugs to treat gonorrhoea. Using surface plasmon resonance (SPR) analysis we have confirmed that TbpA, TdfJ and TdfH were bind to several different classes of glycans as well mannosides which are drugs that mimic mannosyl glycans. Using microscopy, we have also shown that these proteins interact with human genital tract epithelial cells. These data highlight the possibility of targeting these proteins with mannosides to block gonorrhoea-host interactions. Ongoing work is focused on characterizing the role of these mannose-binding proteins during infection using *N. gonorrhoeae* adherence assays with human cervical and urethral epithelial cells in the presence/absence of mannose mimics.

(P161) *GlyCage*: Track cardiovascular risk beyond calendar age using immunoglobulin G

Xingang Li[1], Zhiyuan Wu[1,2], Manshu Song[1], Wei Wang[1]

[1]*Centre for Precision Health, School of Medical and Health Sciences, Edith Cowan University, Perth, Australia,* [2]*Beijing Key Laboratory of Clinical Epidemiology, School of Public Health, Capital Medical University, Beijing, China,*

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We aimed to develop a cardiovascular aging index for tracking cardiovascular risk using IgG N-glycans. In this cross-sectional investigation, we included 1,465 individuals aged 40–70 years from the Busselton Healthy and Ageing Study. We stepwise selected the intersection of altered N-glycans using feature-selection methods in machine learning (recursive feature elimination and penalized regression algorithms) and developed an IgG N-glycosylation cardiovascular age (GlyCage) index to reflect the deviation from calendar age attributable to cardiovascular risk. The strongest contributors to GlyCage index were fucosylated N-glycans with bisecting N-acetylglucosamine (GlcNAc) (glycan peak 6 (GP6), FA2B,) and digalactosylated N-glycans with bisecting GlcNAc (GP13, A2BG2). A one-unit increase of GlyCage was significantly associated with a higher Framingham ten-year cardiovascular risk (odds ratio (OR), 1.09; 95% confidence interval (95% CI): 1.05–1.13) and probability of CVDs (OR, 1.07; 95% CI: 1.01–1.13) independent of calendar age. Individuals with excessive GlyCage (exceeding a calendar age > 3 years) had an increased cardiovascular risk and probability of CVDs, with adjusted ORs of 2.22 (95% CI: 1.41–3.53) and 2.71 (95% CI: 1.25–6.41), respectively. The GlyCage index developed in this study can thus be used to track cardiovascular health using IgG N-glycosylation profiles. The distance between GlyCage and calendar age independently indicates the cardiovascular risk, suggesting that IgG N-glycosylation plays a role in the pathogenesis of CVDs. The generalization of the observed associations and the predictive capability of GlyCage index require external and longitudinal validation in other populations.

(P162) Inhibition of autophagy and ATF6 brunch of UPR blocks pro-metastatic MGAT5-mediated glycosylation of αv-integrins in prostate cancer

Amanda J. Macke[1,2], Artem N. Pachikov[1,2], Taylor E. Divita[1,2], Chad A. LaGrange[3], George P. Hemstreet[3,4], Armen Petrosyan[1]

[1]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA, 68198, [2]The Fred and Pamela Buffett Cancer Center, Omaha, NE, USA, 68198, [3]Division of Urologic Surgery, Department of Surgery, University of Nebraska Medical Center, Omaha, NE, USA, 68198, [4]Omaha Western Iowa Health Care System Urology, VA Service, Department of Research Service, Omaha, NE, USA, 68105,

In the United States, prostate cancer (PCa) is the second-leading cause of cancer lethality in men. Prostate cancer death is attributed to metastatic castration-resistant prostate cancer (mCRPC), driven by α_v integrins. Overexpression of integrins requires domination of N-acetylglucosaminyltransferase-V (MGAT5)-mediated glycosylation over its Golgi competitor, N-acetylglucosaminyltransferase-III (MGAT3). This is a critical event in CRPC as it significantly enhances the retention of α_v integrins at the cell surface of tumor cells and subsequent cluster formation with Galectin-3 (Gal-3). However, the mechanism underlying this phenomenon is missing. Here, we assert that the key to this fact lies in the Golgi disorganization (onco-Golgi) and activation of the ATF6 branch of unfolded protein response (UPR). First, we found that in androgen-responsive PCa cells that exhibit intact Golgi, MGAT3 resides in more proximal Golgi compartments than MGAT5. This explains why in non-malignant cells, integrins are modified by MGAT3 but not MGAT5. However, Golgi dispersal in androgen-refractory PC-3 and DU145 cells results in the mislocalization of MGAT3 from Golgi to the endoplasmic reticulum (ER). In the meantime, MGAT5 retains intra-Golgi localization. This was validated in the tumor samples from patients with PCa and CRPC. Next, we revealed that ATF6-mediated ER stress response maintains a fragmented Golgi phenotype. This, in turn, accelerates Golgi-mediated autophagy, Golgiphagy. Notably, ATF6 depletion not only blocks UPR but also reduces the number of Golgi fragments in both PC-3 and DU145 cells. Inhibition of autophagy by hydroxychloroquine (HCQ) restores compact Golgi and recovers MGAT3 intra-Golgi positioning. Using different sophisticated approaches, including structured illumination superresolution microscopy, lectin chromatography, and immunogold electron microscopy, we found that post-HCQ recovery of Golgi blocks glycan modification via MGAT5 and abrogates delivery of Gal-3 to the cell surface. Paradoxically, Integrin α_v-Gal-3 spots were observed in the medial and terminal Golgi compartments, indicating that the complex between these proteins can be formed en route to the cell surface (right after MGAT5-mediated glycosylation of integrin in Golgi) rather than at the cell surface as was previously suggested. Gal-3 depletion did not affect the total level of Integrin α_v. Still, it reduced PM content via deposition in early endosomes and ER, substantiating Gal-3 is involved in α_v integrins trafficking, but it is not required for their internalization. However, without Integrin α_v, Gal-3 is not internalized and, instead, aggregates at the cell surface. ATF6 depletion and HCQ treatment synergistically decrease Integrin α_v and Gal-3 expression. Finally, in the mice orthotopic tumor model, the combination of ATF6 KD with HCQ had the most noticeable impact on MGAT5-mediated glycosylation and halted metastasis.

(P163) Clinical significance of α2,3-sialylated prostate-specific antigen for prostate cancer in men with elevated PSA level

Tohru Yoneyama[1], Hayato Yamamoto[1], Mihoko Sutoh Yoneyama[2], Shingo Hatakeyama[1], Shintaro Narita[3], Koji Mitsuzuka[4], Akihiro Ito[4], Norihiko Tsuchiya[5], Tomonori Habuchi[3], Chikara Ohyama[1]

[1]Hirosaki University Graduate School of Medicine, [2]Department of Cancer Immunology and Cell Biology, Oyokyo Kidney Research Institute, [3]Department of Urology, Akita University Graduate School of Medicine, [4]Department of Urology, Tohoku University Graduate School of Medicine, [5]Department of Urology, Yamagata University Faculty of Medicine,

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Biomarkers to detect prostate cancer (PC) are needed to reduce unnecessary biopsies. The ratio of α2,3-sialylated prostate-specific antigen (S2,3PSA) to free PSA (S2,3PSA%) in the serum is a potential biomarker of PC. We have developed an automated microcapillary electrophoresis-based immunoassay for S2,3PSA%, which was commercialized in Japan last year. The purpose of this study is to identify the origin of S2,3PSA in prostate tissue and to evaluate the clinical utility of the S2,3PSA% test.

The expression level of sialylation-related genes and S2,3PSA% was analyzed in benign prostate gland and each Gleason pattern (Gp) of cancer tissue in 71 patients who underwent radical prostatectomy at Hirosaki University. To evaluate the clinical significance of S2,3PSA%, we enrolled total 439 patients who underwent prostate biopsy due to PSA levels elevated greater than 4 ng/mL. The primary endpoint was evaluate the specificity of the S2,3PSA% test, total PSA (tPSA) test, and F/T(%)PSA test for the diagnosis of PC based on receiver operating characteristics curve analysis. Secondary endpoints included the validity of the prostate volume correction for S2,3PSA% value (S2,3PSA%density), subgroup analysis by PSA range, and correlation with Grade Group (GG) at biopsy.

The expression level of *ST6GAL1* was significantly decreased in Gp 4 & 5 tissue compared to Gp 3 and benign prostate gland. *ST3GAL3,4,6* was not varied from tumor malignancy. The S2,3PSA% level was also significantly increased in Gp 4 & 5 tissues suggesting that upregulation of the Siaα2,3Gal- ratio on PSA regulated by *ST6GAL1* expression. The AUC of S2,3PSA% for detecting PC was superior to tPSA or F/T(%)PSA (0.7582 vs. 0.6230 and 0.6587, all $P < 0.001$). Furthermore, the specificity of the S2,3PSA% at 90% sensitivity was 37.1%, which was significantly higher than that of the F/T(%)PSA (21.1%) ($p<0.0001$), indicating that the S2,3PSA% was superior to the F/T(%)PSA when used as a secondary test after PSA testing. The positive and negative concordance rates for cancer using the cutoff value at 90% sensitivity as the diagnostic criterion were 68.4% and 71.4% for S2,3PSA%, 62.0% and 52.7% for tPSA, and 63.3% and 58.7% for F/T(%)PSA, respectively, which were higher than those for conventional tests. S2,3PSA%density did not significantly improve the diagnostic accuracy compared to the S2,3PSA%. Subgroup analysis by PSA range showed that the S2,3PSA% values were higher in cancer patients regardless of the PSA range, and were higher than the AUC of PSA and F/T(%)PSA in all PSA ranges. The S2,3PSA% values correlated most positively with GG at biopsy compared with other tests and also showed that the S2,3PSA% values were significantly higher in high-grade cancer patients with GG2 or higher compared with those in biopsy-negative patients.

These results suggest S2,3PSA was mainly secreted in higher-grade PC tissue and the diagnostic performance of S2,3PSA% was superior to conventional strategies.

(P164) Sialyl-Tn antigen regulates the cellular iron homeostasis and the production of extracellular vesicles

Kazuaki Ohtsubo[1], Keisuke Nagao[1], Kasumi Hosomi[1], Tatsutoshi Inuzuka[2], Kento Maeda[3]

[1]*Department of Analytical Biochemistry, Graduate School of health sciences, Kumamoto University, Kumamoto, Japan,* [2]*H.U. Group Research Institute. G.K., Tokyo, Japan,* [3]*Department of Glyco-Oncology and Medical Biochemistry, Osaka International Cancer Institute, Osaka, Japan,*

The tumor microenvironment is composed of various cell types forming a complex multicellular ecological system including divergent functional niches, where these heterogeneous cells communicate via various biogenic manners, such as direct cell-cell interaction, secretion of bioactive molecules, and transfer of extracellular vesicle (EV). Protein glycosylation plays a pivotal role in these processes by modulating molecular interactions and cellular responses by finetuning glycan structures. In various cancers, early termination of the *O*-glycan synthesis is occasionally observed, in which Sialyl-Tn (sTn) antigen is generated. Its expression is well-associated with metastasis and poor prognosis and thereby is applicated to a clinical tumor marker. We have recently found that sTn antigen-expressing cells produce a larger number of EVs than non-expressing cells. Consistent with this, the sTn antigen-expressing cells abundantly expressed tumor suppressor-activated pathway 6 (TSAP6), which is a regulator of the EV-production; however, its detailed function in EV production is unknown. The genetic attenuation of TSAP6 resulted in the reduction of the EV production in the sTn antigen-expressing cells. These results indicated that the sTn antigen upregulated TSAP6 expression and enhanced EV production.

TASP6 is known as a ferrireductase participating in iron homeostasis by reducing Fe^{3+} to Fe^{2+}. Consistent with the upregulated TSAP6 expression, total iron levels, and mitochondrial Fe^{2+} levels were significantly increased in the sTn antigen-expressing cells. Since mitochondrial Fe^{2+} is indispensable for the synthesis of iron-sulfur clusters, which mediate electron transfer within the respiratory chain, the sTn antigen-expressing cells showed elevated mitochondrial membrane potential. This was significantly reduced by the treatment of an iron-chelating agent, Deferoxamine. Interestingly, the cellular iron levels affected cellular EV production levels. These results suggested that the sTn antigen-induced TSAP6 is involved in cellular energy metabolism and also in EV production through cellular iron homeostasis.

The enhanced EV production in the sTn antigen-expressing cells substantially augmented the delivery of EVs to the recipient cells. The cells receiving the EVs from the sTn antigen-expressing cells showed increased cellular motility, which was coincident with an elevation of cellular focal adhesion kinase (FAK) protein levels. Besides, the inhibition of FAK significantly reduced the motility of the EV-recipient cells. Moreover, the EVs produced by the sTn antigen-expressing cells selectively and abundantly encased FAK protein. These results indicated that the sTn antigen-expressing cells transferred FAK protein to the recipient cells via EVs and enhance their motility. These findings would contribute to facilitating the elucidation of the pathophysiological significance of the sTn antigen in the tumor microenvironments and tumor development.

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(P165) EZH2 is a Novel Epigenetic Regulator of Heparan Sulfate Biosynthesis

Neil Patel[1], Amrita Basu[1,2,3], Emily Spector[1,2], Rachel Champagne[1,2], Elijah Nicholson[1,2,3], Ryan Weiss[1,2,3]

[1]*University of Georgia,* [2]*Complex Carbohydrate Research Center, University of Georgia,* [3]*Department of Biochemistry and Molecular Biology, University of Georgia,*

Heparan sulfate proteoglycans (HSPGs) are expressed on virtually all animal cells and in the extracellular matrix. Each HSPG consists of a core protein with one or more covalently attached long, linear heparan sulfate (HS) chains. These HS chains are composed of alternating glucosamine and uronic acids that are heterogeneously *N*- and *O*-sulfated, enabling electrostatic interactions with various extracellular signaling molecules and other ligands. The fine structure of the HS chains mediates a host of cellular interactions which are crucial to maintaining proper cell growth and homeostasis. Dysregulation of HS expression and assembly has been implicated in the progression of various cancers, including melanoma. Recently, our lab has shown that the regulation of SULF1, an extracellular HS 6-O sulfatase, can attenuate melanoma cell growth *in vitro*. In this study, we aimed to understand the epigenetic regulatory mechanisms of HS biosynthesis and how these regulatory mechanisms may be involved in the progression of melanoma growth. To investigate this, we conducted an *in silico* analysis of the promoters of all genes involved in HS assembly using web-based bioinformatic tools and publicly available chromatin immunoprecipitation datasets. Intriguingly, enrichment analysis revealed multiple members of the polycomb repressive complex 2 (PRC2), a prominent set of chromatin remodeling proteins essential for normal development which are frequently mutated in cancer, as potential regulators of HS biosynthesis. To investigate the epigenetic regulation of HS biosynthesis, we targeted the primary catalytic subunit of PRC2, the histone methyltransferase enhancer of zeste 2 (EZH2), which is commonly overexpressed in melanoma and associated with poor prognosis and formation of metastases, in human melanoma cells. We found that EZH2 regulates the expression of multiple enzymes involved in HS biosynthesis to modify the fine structure of HS chains, which has downstream effects on HS-ligand interactions and cell homeostasis. Pharmacological inhibition of EZH2-mediated histone methylation and targeting other prominent members of the PRC2 revealed non-canonical regulatory mechanisms for controlling HS assembly. Overall, these studies provide insight into the molecular mechanisms by which HS biogenesis is differentially regulated in physiology and disease and may provide insight regarding potential therapeutic targets for melanoma therapy.

(P166) Role of Truncated O-glycans in Pancreatic Cancer Progression and Metastasis

Satish Sagar[1], Yasuyuki Matsumoto[2], Suttipong Suttapitugsakul[2], Michael A. Hollingsworth[1], Richard D. Cummings[2], Prakash Radhakrishnan[1]

[1]*Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, NE,* [2]*Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA,*

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease. A fundamental discovery in this area is the finding that >80% of PDACs express surface glycoproteins with abnormal truncated O-glycans, the Tn antigen (CD175 - GalNAcα1*O*-Ser/Thr) and/or its sialylated form STn (CD175s - Siaα2-6GalNAcα1*O*-Ser/Thr). Their expression correlates with poor prognosis and reduced patient survival. STn overexpression, which is always accompanied by Tn expression, occurs in many epithelial cancer cells, with the highest frequency in PDAC. We demonstrated that increased Tn/STn expression in PDAC patients was partly due to hypermethylation/silencing of *COSMC*, the gene encoding the Core 1 Specific Molecular Chaperone. Cosmc is a unique molecular chaperone in the endoplasmic reticulum required for folding a single client, the T-synthase, whose expression is required to generate normal, extended core 1 O-glycans. Targeted deletion of *COSMC* induces Tn/STn antigens on cancer cell surfaces and increases the tumorigenicity of human PDAC cells. However, *Cosmc* knockout-induced Tn/STn antigens mediated disease progression, and metastasis is unknown. Recently, we deleted *Cosmc* in the KPC mouse model (LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; *Cosmc*$^{fl/fl}$; Pdx-1^{Cre}) to investigate the role of Tn antigen during PDAC development and progression. Loss of *Cosmc* in KPC mice induces aberrant expression of Tn/STn antigens, leading to the development of early precancerous lesions (∼4 weeks), PDAC (∼4 weeks), metastasis (∼8 weeks), and reduced animal survival compared to parental KPC mice. KPC-*Cosmc*^{KO} PDAC cells exhibited increased cell proliferation, migration, invasion, colony formation, and EMT characteristics compared to KPC cells. Also, the appearance of Tn/STn antigen promotes PDAC tumorigenesis via constitutive activation of epidermal growth factor receptor (ErbB1/EGFR) and its downstream PI3K/AKT signaling cascades in KPC-*Cosmc*^{KO} cells and tumors. Further, treatment of Tn^{+} Cosmc^{KO} tumor cells and tumor-bearing animals with a novel afucosylated anti-Tn monoclonal antibody (mAb; aRemab6) inhibits *in vitro* and *in vivo* tumor growth via complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), respectively.

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(P167) Anti-androgen therapies upregulate Siglec ligands to maintain prostate tumour immune suppression

Rebecca Garnham[1], Daniel Geh[1], Ryan Nelson[1], Edward Schmidt[2,3], Laura Walker[1], Craig Robson[1], Matthew Macauley[2,3], Jennifer Munkley[1], Jack Leslie[1], Emma Scott[1]

[1]*Newcastle University, Centre for Cancer, NE1 3BZ, UK,* [2]*Department of Chemistry, University of Alberta, Edmonton, AB, T6G 2G2, Canada,* [3]*Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, T6G 2E1, Canada,*

Trials of immune checkpoint blockade for the treatment of advanced prostate cancer have not yet yielded a robust anti-cancer response, primarily due to the immune-suppressed microenvironment within prostate cancer tumours. The involvement of ST3Gal1 and other sialyltransferases in cancer and immune suppression is evident through the production of immunosuppressive sialoglycans, which act as binding molecules for Siglec receptors. The precise mechanisms governing the synthesis of Siglec ligands remain unclear, and our understanding of how the sialoglycan-Siglec axis contributes to prostate cancer's ability to evade anti-tumour immune responses is limited.Our research has demonstrated a negative correlation between ST3Gal1 levels and androgen signalling in prostate tumours. Through experiments involving mouse models, patient samples, and in vitro studies, we have demonstrated that ST3Gal1 is responsible for generating sialoglycans that can interact with the Siglec-7 and Siglec-9 immunoreceptors. This interaction effectively hinders the immune system's ability to clear cancer cells. Furthermore, we have profiled Siglec-7/9 ligands and their corresponding immunoreceptors in prostate tumours.Of particular significance is our finding that these interactions can be influenced by enzalutamide, an anti-androgen therapy, potentially perpetuating immune suppression in enzalutamide treated tumours. We conclude that the function of ST3Gal1 plays a pivotal role in immune checkpoint blockade resistance in prostate tumours. This insight provides a solid foundation for considering the implementation of therapeutic strategies targeting glyco-immune checkpoints in the context of advanced prostate cancer.

(P168) Signature human melanoma cell surface glycans license vascular adhesion through galectin-3

Jose Souchak, III[1], Basilio E. Garcia[1], Norhan B.B. Mohammed[1], Lee Seng Lau[1], Charles J. Dimitroff[1]

[1]*Translational Glycobiology Institute at FIU (TGIF), Department of Translational Medicine, Herbert Wertheim College of Medicine, Florida International University, Miami, FL,*

Melanoma is a relatively rare yet highly lethal form of skin cancer, and despite recent therapeutic advances, only ~30% of patients with metastatic melanoma (MM) survive beyond five years. Understanding the molecular mechanisms that drive melanoma cell systemic dissemination and extravasation is fundamental for predicting metastatic potential and developing novel anti-melanoma therapies. Previous studies revealed that human MM cell surfaces, unlike normal melanocytes, possess an abundance of linear poly-N-acetyllactosaminyl glycans (poly-LacNAcs) characteristically recognized by a family of β-galactoside-binding proteins known as galectins (Gal). We hypothesized that circulating galectins, namely Gal-3 and -8, which have been functionally associated with melanoma progression, bind to MM cell surface poly-LacNAcs, facilitating cell "bridging" to vascular endothelial cells (ECs) to initiate tumor cell adhesion and subsequent extravasation into distant tissues. Using flow cytometry, we verified the Gal-3 and -8-binding activity on MM cell and human vascular EC surfaces. We also revealed the absence of the canonical vascular endothelial (E)-selectin-binding glycans, including sialyl Lewis X and A, on MM cells, implicating Gal-3 and Gal-8 as alternative cell adhesion mechanism(s). Consistently, using RT-qPCR, we demonstrated the lack of expression of fucosyltransferases enzymes (FT3-7), which are crucial for the formation of sialyl Lewis X and A that mediate E-selectin-based adhesion. Additionally, we conducted parallel-plate chamber assays to simulate the physiological shear stress of circulating MM cells flowing over human ECs. Data from these experiments revealed that exogenous Gal-3 significantly enhanced MM cell adhesion to ECs compared to untreated or negative Gal-3 inhibitor control groups. These findings support our premise that MM cells may use a galectin-based model as an alternative mechanism for metastatic spread and distinct from the established dependence on vascular E-selectin. Importantly, this study implies that galectins and their ligands on MM cell surfaces can be targets for innovative therapeutics tailored to melanoma patients who commonly show higher levels of Gal-3 and Gal-8.

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(P169) Improvement of Antibody Activity by Controlling its Dynamics using Glycan-Lectin Interaction

Kazuya Kabayama[1,2,3], Yoshiyuki Manabe[1,2,3], Yuki Iizuka[1], Ryuku Yamamoto[1], Keita Ito[1], Kanae Hatano[1], Koichi Fukase[1,2,3]

[1]*Department of Chemistry, Graduate School of Science, Osaka University,* [2]*Forefront Research Center, Graduate School of Science, Osaka University,* [3]*Institute for Radiation Sciences, Osaka University,*

Antibody dynamics on membranes, such as endocytosis and clustering, are vital in determining antibody functions. In this study, we demonstrated that glycan conjugation can modulate antibody dynamics through the glycan-lectin interaction to regulate its potency. The anti-HER2 antibody, an anti-breast cancer antibody, was conjugated with galactose-containing N-glycan, and its internalization was suppressed by interaction with galectin-3, leading to enhanced complement-dependent cytotoxic (CDC) activity. This glycan- antibody conjugate is proposed as a new approach to modulate antibody activity and may provide an alternative strategy for redeveloping antibody drugs that do not exhibit sufficient activity.

(P170) Targeting altered glycosylation for broad cancer detection

Matthew R. Kudelka[1,2,4,5,†], Wei Gu[2,6,†], Yasuyuki Matsumoto[2,7], Tongzhong Ju[1,7], Richard H. Barnes, II[2,8], Robert J. Kardish[2,9], Jamie Heimburg-Molinaro[2], Sylvain Lehoux[2,10], Junwei Zeng[2,11], Cynthia Cohen[3], Brian S. Robinson[3], Kinjal S. Shah[3,12], Elliot L. Chaikof[2], Sean R. Stowell[3,13,‡], Richard D. Cummings[2,‡,*]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30033, United States,* [2]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States,* [3]*Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30033, United States.,* [4]*Present addresses: Robin Chemers Neustein Laboratory of Mammalian Cell Biology and Development, The Rockefeller University, New York, NY, United States,* [5]*Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY,* [6]*BeiGene Institute, BeiGene R&D, Shanghai, China,* [7]*Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, MD, United States* [8]*Penrose TherapeuTx, Plymouth, MI, United States,* [9]*Safeguard Biosystems, Inc., Guelph, Ontario Canada,* [10]*NovAb Inc., Atlanta, GA, United States,* [11]*Medical Research Institute, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangzhou, China,* [12]*Department of Pathology and Laboratory Medicine, University of Tennessee Health Science Center, College of Medicine,* [13]*Joint Program in Transfusion Medicine Brigham and Women's Hospital, Harvard Medical School, Boston, MA,*

*Corresponding author: Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

†These are the co-first authors.
‡These are the co-senior authors.

There is an urgent need for universal biomarkers for early cancer detection. Most cancer deaths arise from metastasis and could be avoided by early detection. Current protein-based biomarkers only detect a few tumor types because of tissue-specific gene expression. In contrast, carbohydrate alterations can occur across tumor types, offering universal detection. We previously generated a specific recombinant antibody against Tn, a near universal tumor-associated carbohydrate antigen. Here, we develop an anti-Tn antibody microarray (ATAM) for near universal cancer detection, printing anti-Tn IgG1 Remab6 and probing with anti-Tn IgM ReBaGs6. We demonstrate picogram level detection of Tn in defined glycoproteins and complex biologic samples, including stool from a genetically engineered mouse and serum from *COSMC*-deleted human cells, but not in endogenous IgA1. Further, we stain hundreds of human tumors and controls from diverse tissues, confirming tumor-specific Tn expression in over half of most tumors. Our ATAM is a promising technology for early near universal cancer detection.

(P171) Radical Fringe Promotes Ovarian Cancer Growth and Drives Notch-Mediated Expression of Proteins that Control Cancer Stem Cells and Immune Suppression

Zhongpeng Lu[3,†], Kenjiroo Matsumoto[2,†], Natalie Trent[1], Jason Rodriquez[1], Victor Carvajal[1], Robert Remy[1], Allison Winchester Amado[1], Demi Burns[1], Cayla Plummer[1], Sravika Kayithi[1], Kevin Reilly[1], Robert S. Haltiwanger[2,], Karen L. Abbott[1,+]

[1]*Arkansas Colleges of Health Education, Oncology Research Center, Department of Biochemistry and Cell Biology, Fort Smith, AR, USA,* [2]*Complex Carbohydrate Research Center, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA,* [3]*Department of Translational Medicine, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA,*

†These authors contributed equally and +these principal investigators supervised the research equally

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Ovarian cancer is often diagnosed at later stages leading to higher rates of recurrent disease and chemoresistance making the identification of new therapeutics a priority. The Notch signaling pathway plays a prominent role in tumorigenesis and represents an important underexplored pathway for ovarian cancer therapeutic development. O-fucose is a major component of the glycosylation present on Notch receptors that modulates signal transduction of this pathway. In particular, the Fringe enzymes extend O-fucose on Notch EGF repeats modulating molecular interactions with the ligands Delta and Jagged. Radical Fringe (RFNG) is amplified in a large number of ovarian cancer patients and is associated with reduced overall survival. In this study, we use mass spectrometry to analyze the O-fucose glycosylation on endogenous Notch 1 receptors confirming the loss of RFNG-mediated glycosylation in our *RFNG*-KO ovarian adenocarcinoma cell lines. Our i*n vivo* xenograft model demonstrates that RFNG activity promotes ovarian tumorigenesis. We show that RFNG expression activates Notch signaling and controls the levels of key Notch regulated proteins that have roles in cell cycle control (CDK6), stem cell maintenance (GPC3), and immune suppression functions (CD33, SELL, and CD47). Therefore, we conclude that RFNG is a target for the development of novel therapeutics that can block tumor growth and remove immune suppression.

(P172) Machine-learning model based on glycosyltransferase expression predicts multiple cancer types, subtypes, and survival probability

Jing Kai[1], Luyao Yang[2], Ayman F. AbuElela[1], Alyaa M. Abdel-Haleem[2], Asma S. AlAmoodi[1], Abdulghani A. Bin Nafisah[3], AlFadel M. AlShaibani[3], Ali S. AlZahrani[3], David Gomez-Cabrero[1], Xin Gao[2,4], Jasmeen Merzaban[1,4]

[1]Bioscience Program, King Abdullah University of Science and Technology (KAUST), Biological and Environmental Sciences and Engineering (BESE) division, Thuwal, 23955-6900, Saudi Arabia, [2]Computer Science Program, King Abdullah University of Science and Technology (KAUST), Computational Bioscience Research Centre (CBRC), Thuwal, 23955-6900, Saudi Arabia, [3]Department of Molecular Oncology, King Faisal Specialist Hospital, and Research Center, Riyadh, 11211, Saudi Arabia, [4]KAUST Smart-Health Initiative, King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia,

Existing methods for diagnosing cancer, such as histology and imaging for specific markers, tend to vary depending on the variety of the disease, as is the case for the PAM50 whose primary clinical use is to subtype breast cancers. Our research describes a one-stop cancer classification tool capable of accurately differentiating and identifying 27 types of cancer and their subtypes. Created using machine learning of thousands of tissue samples drawn from the Cancer Genome Atlas and verified against both independent databases and patient samples, the classifiers reveal that each cancer type and subtype has a unique and highly precise glycosyltransferase (GT) signature. These signatures can be quickly identified from unlabelled tissue, revealing more than nine times out of 10 whether the sample is cancerous, which type of cancer it is, and which sub-variety it is. Our cancer classifiers open a new frontier in using the precision of glycan expression patterns to diagnose cancer because, as we demonstrate, a subset of GT genes are almost universally present as the disease spreads, and therefore have a functional role in cancer's destructiveness. The broad scope of the tool doesn't compromise accuracy, either; our breast cancer classifier differentiates between breast cancer subtypes with up to 92% accuracy, a vast improvement on the most widely used industry test, the PAM50. By streamlining the process of diagnosis, the implications for pan-cancer diagnostics and prognosis are significant both in time and cost savings. One classifier can even predict the probability of survival for patients diagnosed with glioma, by zooming in on four GT genes that are strongly related to prognosis. Our experiments demonstrate the benefit of adding machine learning to typical gene expression analysis and outline the potential of GT genes as diagnostic and prognostic cancer biomarkers.

(P173) Core fucose is involved in the redox system and is a potential biomarker of lung cancer

Yuki Ohkawa[1], Kanto Noriko[1], Miyako Nakano[2], Yasuhiko Kizuka[3], Yoshiki Yamaguchi[4], Yoichiro Harada[1], Eiji Misyoshi[5], Jianguo Gu[6], Naoyuki Taniguchi[1]

[1]Department of Glyco-Oncology and Medical Biochemistry, Osaka International Cancer Institute, [2]Graduate School of Integrated Sciences for Life, Hiroshima University, [3]Institute for Glyco-core Research (iGCORE), Gifu University, [4]Division of Structural Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Medical and Pharmaceutical University, [5]Department of Molecular Biochemistry and Clinical Investigation, Graduate School of Medicine, Osaka University, [6]Division of Regulatory Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Medical and Pharmaceutical University,

Core fucose represents a fucose moiety that is attached to the innermost GlcNAc of *N*-glycans. Our research group identified a glycosyltransferase: α1,6-fucosyltransferase (FUT8) and reported its functional machinery for the generation of the core fucose structure about 25 years ago. After then, many groups including us continue the efforts to uncover the roles of core fucose and/or FUT8 in human diseases. Herein, we focused on two glycoproteins: superoxide dismutase 3 (SOD3) and immunoglobulin G (IgG) to show the molecular aspects of core fucose in lung cancer.

Superoxide dismutases (SODs) are enzymes that convert superoxide radicals into hydrogen peroxide and oxygen. SOD3 is an extracellular superoxide dismutase and contains a single *N*-glycan chain. Firstly, we performed the structural analysis of

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N-glycans in SOD3 in the human sera and found that the fucose structure of SOD3 was increased in the patients with lung cancer. In cell lines of non-small lung cancer cell (NSCLC), we also found a high level of the core fucose structure in SOD3, as determined by lectin blotting and mass spectrometry. To address the roles of the core fucose structure of SOD3, we generated *FUT8* gene knockout lung cancer cells. Using these cells, we found that the core fucose structure of SOD3 was required for its secretion and enzymatic activity, which contributes to the suppression of cell growth of NSCLC cells. These data demonstrate that *N*-glycans, especially those with core fucose structures, regulate the anti-tumor functions of SOD3 against NSCLC.

On the other hand, IgG is one of the most abundant proteins in human sera. IgG also carries a single *N*-glycan chain in its constant region and the structural signature of *N*-glycan in IgG is targeting to develop the novel biomarkers in these years. In this study, we developed a highly specific monoclonal antibody (mAb) against core fucose of human IgG (core fucosylated IgG). By ELISA and latex turbidimetric immunoassay using this mAb, we found the decreased level of core fucosylated IgG in the sera of patients with lung cancer. In a co-culture analysis using human lung cancer cells and antibody-secreting B cells, the downregulation of the *FUT8* gene and a low level of core fucoselated IgG in antibody-secreting B cells were observed. A dramatic alteration in gene expression profiles for cytokines, chemokines, and their receptors were also observed after co-culturing, and we found that the C-C motif chemokine 2 (CCL2) was partially involved in the downregulation of the *FUT8* gene in antibody-secreting B cells. These results suggest that communication with CCL2 between lung cells and antibody-secreting B cells downregulate the level of core fucose of the *N*-glycan in IgG, i.e., the increased level of acore fucosylated (non-core fucosylated) IgG.

(P174) N-glycome profiling of progression and immune cell clusters across colorectal carcinoma using MALDI-MSI and MALDI-IHC

Lyndsay Young[1], Paul Nietert[3], Rachel Stubler[4], Caroline G. Kittrell[1], Grace Grimsley[1], David N. Lewin[5], Chadi Hajar[5], Katherine Wang[5], Elizabeth O'Quinn[2,5], Kristin Wallace[2,5], Richard R. Drake[1,2]

[1]Department of Cell and Molecular Pharmacology and Experimental Therapeutics, College of Medicine, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA, [2]Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC, 29425, USA, [3]Department of Public Health Sciences, College of Medicine, College of Medicine, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA, [4]Department of Biochemistry and Molecular Biology, College of Medicine, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA, [5]Department of Pathology and Laboratory Medicine, College of Medicine, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA,

Colorectal cancer (CRC) is a leading cause of death worldwide, often developing from defined genetic mutations via pre-cancerous adenomas to adenocarcinomas. Early detection through regular screening results in a 5-year survival rate for 90% of patients. Unfortunately, only a fraction of CRC cases are found in pre-invasive stages and progression can occur silently over 10–15 years. The intricate interplay between the immune system and tumor cells within the tumor microenvironment plays a pivotal role in CRC progression. Immune cell clusters can either inhibit or facilitate tumor initiation, growth, and metastasis. To better understand this relationship, we conducted N-glycomic profiling via MALDI-MSI and assessed cell composition using MALDI-IHC. First, we employed MALDI-MSI to identify the N-glycome of normal colon specimens, pre-invasive lesions, and cancerous tissues in various CRC stages. 174 N-glycan species were detected across all samples revealing a shift in N-glycome profiles from normal to cancerous tissues, marked by a decrease in high mannose N-glycans. Further analysis of precancerous to invasive carcinomas revealed increases in pauci mannose and tetra-antennary N-glycans with stage, and a distinct stratification in the N-glycome profile between non-mucinous and mucinous CRC tissues driven by high mannose, bi-antennary and fucosylated N-glycans. Next, we employed multiplexed MALDI-IHC using photocleavable mass tagged- antibodies for CD3, CD8, CD11b, CD20, and CD68 immune markers and E-cadherin, collagen 1A1, and actin αSmooth muscle extracellular matrix components to probe the cellular composition of the tumor microenvironment. Approximately 60% of the immune clusters were CD20+ B-cells with additional colocalization of 8% of CD3+ T-cells in precancerous lesions. These immune clusters also emerged with a distinct and predictive profile of bisecting and branched N-glycans by MALDI-MSI. This innovative, two dimensional approach provides additional information about the cellular composition of the tumor microenvironment, and sheds light on the complex mechanisms underlying CRC.

(P175) Spatial-Omics reveals that cancer-associated glycan changes occur early in liver disease development in a western-diet mouse model of MASLD

Shaaron Ochoa-Rios[1], Stéphane Grauzam[1], Rebecca Gregory[1], Peggi M. Angel[1], Richard Drake[1], Kristi Helke[1], Anand Mehta[1]

[1]Medical University of South Carolina,

Metabolic dysfunction-associated liver disease (MASLD) is a progressive disease and comprises different stages of liver damage. While some of these are considered benign, untreated MASLD can progress to life-threatening end-stage conditions such as

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cirrhosis and liver cancer. The increased rates of liver cancer related to MASLD have now become a public health concern and an understanding of molecular factors driving this disease is urgently needed. N-linked glycan alterations have been established to be signatures in liver diseases, specifically cirrhosis, and liver cancer. Similarly, previous reports by our group demonstrate that significant core fucosylation occurs in serum and directly in human liver tissue of HCC and MASLD patients. However, the N-liked glycan changes during the progression of MASLD up to liver cancer are still unknown. Here, we induced different stages of MASLD (using a Western diet model) and liver cancer-related phenotypes (using a diet and carcinogen model). We elucidated the N-glycome profile at the different stages of the disease and identified specific N-glycan structures including fucosylated and highly branched N-linked glycans, at very early stages of liver injury (month 4) which in humans are associated with cancer development. In addition, we report that N-linked glycan alterations can be observed in our models before liver injury is identified in histological analysis. Overall, we propose the use of N-glycosylation alterations as a promising biomarker for the early diagnosis of liver injury and demonstrate the importance of further exploring these alterations in an in vivo model for mechanistic studies.

(P176) Serum glycoproteomics for diagnosing and staging epithelial ovarian cancer

Prasanna Ramachandran[1], Chirag Dhar[1], Gege Xu[1], Chad Pickering[1], Tomislav Čaval[1], Rachel Rice[1], Daniel Serie[1], Flavio Schwarz[1]

[1]*InterVenn Biosciences, South San Francisco, USA,*

Epithelial ovarian cancer (EOC) is one of the most common types of gynecologic malignancies. Symptoms of early stage EOC may be vague and nonspecific, making early diagnosis of EOC challenging. Minimally invasive methods are needed for early diagnosis of EOC. Glycoconjugates play important roles during the progression of ovarian and other cancers. Glycoproteins expressed by ovarian cancer cells allow the tumor cells to circumvent immune responses and play an important role in tumor metastasis. Circuiting serum glycoproteins are a rich source of biomarkers for EOC. In this study, we applied a novel blood-based high throughput glycoproteomic platform to identify glycopeptide biomarkers that differentiate between individuals with benign pelvic masses and malignant EOC. We used a subset of these markers to generate a classifier that distinguished benign pelvic tumors and EOC with sensitivity and specificity of 83.5% and 90.1% in the training set, and 86.7% and 86.7% in the testing set, respectively. We also observed that patients with late-stage EOC (FIGO stage III and IV) had higher levels of tri- and tetra-antennary glycopeptide markers containing fucose. We selected a subset of these markers to build a classifier that differentiated between early- and late-stage EOC. Lastly, we detected a similar upregulation of fucosylated glycans and gene expression signatures suggestive of multi-antennary glycans in late-stage EOC tissues. In summary, we identified biomarkers that differentiated between benign and malignant pelvic masses, or between early- and late-stage EOC, and detected comparable glycosylation signatures in circulating factors and tumor tissues Our results provide preliminary evidence that point to the feasibility of mass spectrometry-based serum glycoproteomics for developing clinically actionable liquid biopsy tests to diagnose and stage patients with EOC.

(P177) Regulation of microglia and macrophage activity by the polysialic acid-Siglec axis

Hauke Thiesler[1], Lina Gretenkort[1], Katharina Petzold[1], Iris Röckle[1], Leonie Hoffmeister[2], Herbert Hildebrandt[1]

[1]*Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany,* [2]*Institute of Clinical Chemistry, Hannover Medical School, Hannover, Germany,*

Through interactions with Siglec immune receptors, the linear glycan polymer polysialic acid (polySia) is implicated in the control of microglia and macrophage activity. In cultured murine microglia, Siglec-E mediates the inhibition of inflammatory activation achieved by the application of protein-free polySia or conveyed by a negative feedback loop with polySia attached to the protein carriers NRP2 and ESL-1, accumulating in the Golgi compartment and released through ectodomain shedding upon proinflammatory activation [1–2]. Generation of polySia fractions with defined degrees of polymerization (DP) enabled us to demonstrate that polySia with a DP of at least 24 sialic acid residues is needed for Siglec-E-mediated microglia inhibition, and application of polySia DP24-30 to a murine slice culture model of myelin repair improves regeneration through Siglec-E-dependent microglia regulation [3]. Unlike in mice, the inhibitory polySia receptor Siglec-11 on human microglia and macrophages has an opposing, activating paired receptor, Siglec-16, but due to a nonfunctional *SIGLEC16P* allele, *SIGLEC16* penetrance is less than 40%. The neuropathological relevance of Siglec-16 was demonstrated in a recent study showing increased survival of glioblastoma patients with a functional polySia-Siglec-16 axis linked to proinflammatory activation of tumor-associated microglia and macrophages (TAM) [4]. Unresolved issues include the regulation of Siglec-16 on TAM. We therefore started to analyze polySia and Siglec-16 expression and trafficking in human THP-1 macrophages and during TAM differentiation of freshly isolated PBMCs from healthy donors with different *SIGLEC16* gene status. Similar to previous findings for Siglec-E [2], first results indicate that cell surface levels of Siglec-16 are low, because the receptor is rapidly internalized in response to polySia exposure. This may explain, why it is so difficult to study Siglec-16 by immunostaining.

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Funding: Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project number 409784463 (DFG grant HI 678/10-2, FOR2953 to H.H.)

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(P178) Polysialic acid release by tumor-associated microglia and macrophages

Hauke Thiesler[1], Jannik Schwabe[1], Alina Ottlewski[2], Iris Röckle[1], Christian Hartmann[3], Kerstin Schwabe[2], Herbert Hildebrandt[1]

[1]Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany, [2]Department of Neurosurgery, Hannover Medical School, Hannover, Germany, [3]Department of Neuropathology, Institute of Pathology, Hannover Medical School, Hannover, Germany,

Through interactions with Siglec immune receptors, the linear glycan polymer polysialic acid (polySia) is implicated in immune regulation of the tumor microenvironment. Recently, we demonstrated that increased survival of glioblastoma patients is linked to proinflammatory activation of tumor-associated microglia and macrophages (TAM) caused by interactions between polySia attached to its major protein carrier NCAM on tumor cells and Siglec-16 on TAM [1]. However, as shown *in vitro*, polySia can also be produced by activated microglia and macrophages. In this case, polySia is attached to the protein carriers NRP2 and ESL1. These polysialylated proteins accumulate in the Golgi compartment before being released through ectodomain shedding [2,3]. To address the unresolved question if activated TAM are able to shed polysialylated proteins *in* vivo, we exploited an autologous rat glioma model [4]. PolySia-negative BT4Ca rat glioma cells were inoculated into the frontal cortex of two groups of BDIX rats. One received surgical tumor resection after eight days. Both groups were kept until the approved humane endpoint criterion. Immunohistology revealed numerous tumor-infiltrating IBA1-positive microglia and macrophages and a gradient of microglia accumulation and activation towards the tumor border in the surrounding brain parenchyma. A small population of IBA1-positive cells with activated morphology and intracellular, Golgi-like polySia staining was detected around and within the tumor, while larger amounts of non-cell-associated polySia appeared in the glial scar-like brain tissue adjacent to the tumor. Notably, polySia serum levels were elevated in tumor-bearing rats without surgical intervention. These findings seem consistent with the shedding of polysialylated proteins from TAM activated in response to the tumor insult.

Funding: Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project numbers 324633948 and 409784463 (DFG grants HI 678/9-3 and HI 678/10-2, FOR2953 to H.H.)

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(P179) Serologic LC-MS/MS-PRM of IgG N-glycoform G0FN and sialylated HPX O-glycoforms effectively identifies progressing liver fibrosis

Aswini Panigrahi[1,2], Lihua Zhang[1,2], Julius Benicky[1,2], Chiranjeev Dash[1], Jaeil Ahn[3], Radoslav Goldman[1,2,4]

[1]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20057 USA, [2]Clinical and Translational Glycoscience Research Center, Georgetown University Medical Center, Georgetown University, Washington, DC 20057 USA, [3]Department of Biostatistics, Bioinformatics and Biomathematics, Georgetown University, Washington, DC 20057 USA, [4]Department of Biochemistry and Molecular and Cellular Biology, Georgetown University, Washington, DC 20057 USA,

There are more than 1 billion people with some form of chronic liver disease (CLD), that resulted in 1.32 million deaths in 2017 and non-invasive detection of a progressing liver fibrosis is a well-documented unmet healthcare need. To address this need, we introduced serologic quantification of sialylated O-glycoforms of the liver secreted protein HPX and a galactosylated N-glycoforms of immunoglobulin G. Here we show that an optimized targeted microflow LC-MS/MS-PRM method (5 min per run) using an isotopically labeled glycopeptide standard accurately quantifies the glycopeptide biomarkers in unfractionated serum samples. We used the assay to screen 304 serum samples, that includes 79 controls, and progressive fibrotic liver disease groups of HCV etiology (43, 87 and 88 samples of biopsy verified mild, moderate, and advanced fibrosis, respectively). The results validated that abundance of the IgG N-glycoform G0FN [(HexNAc(5)Hex(3)Fuc(1)] is significantly ($P < 0.0001$) higher in sera of mild fibrosis patients compared to the control group. The S-HPX, i.e. the ratio of disialoT to monosialoT antigen

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[(HexNAc(1)Hex(1)Neu5Ac(2) over HexNAc(1)Hex(1)Neu5Ac(1)] efficiently detected significant fibrosis (mild to moderate $P < 0.0001$, moderate to advanced $P < 0.0001$). In summary, our assays provide for a rapid and effective serologic screen of liver fibrosis which has clinical potential and should be further evaluated in fibrotic liver disease of various etiologies.

(P180) Glyco-immune Checkpoint Therapies Targeting the Hypoxia Marker CAIX

Brett Garabedian[1], James Paulson[1]

[1]*Department of Molecular Medicine, and Department of Immunology & Microbiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States,*

Tumor hypoxia is a shared feature among solid cancers that drives proliferation and hinders clinical treatment. The hypoxia marker Carbonic Anhydrase IX (CAIX) is a key contributor, acidifying the tumor microenvironment and creating an immunosuppressive niche. The cancer cell surface is additionally characterized by highly abundant sialic acid containing glycans that suppress immune activation by engaging inhibitory Siglecs on tumor infiltrating leukocytes. Established methods to restrict tumor growth separately include (i) chemical inhibition of CAIX to limit tissue acidosis and (ii) sialic acid degradation to polarize immune cells toward inflammatory subsets. By conjugating inhibitory ligands of the hypoxia marker CAIX (**CAIX*i***) to sialidase (**S**), we have developed bifunctional therapeutics, termed **CAIX*i*-S**, capable of targeting both axes simultaneously.

Sialidase was rationally engineered to afford a suite of CAIXi-S conjugates spanning a range of activities, allowing for synergy through robust CAIX inhibition that is enhanced through a finely tuned sialidase payload. A short sialidase serum half-life of <1h motivated pharmacokinetic optimization through genetic fusion to an albumin-binding nanobody, affording a t1/2≥24h. PK-optimized **CAIX*i*-S**∗ retain nanomolar binding to CAIX while selectively degrading sialic acids from CAIX+ tissues *in vitro* and *in vivo*. Evaluation of CAIX***i***-S∗ is underway in syngeneic models of renal, breast and colon cancer using experimental methods that include survival analysis, flow cytometry and spatial transcriptomics. We anticipate CAIXi-S∗ will enhance anti-tumor immunity by simultaneously (i) inhibiting CAIX activity to alleviate tumor acidosis and (ii) degrading sialoglycan ligands of inhibitory Siglecs to collectively invigorate immune responses beyond either single approach.

(P181) A 6-O-endosulfatase Activity Assay Based on Synthetic Heparan Sulfate Oligomers

Julius Benicky[1], Jian Liu[2], Zhangjie Wang[3], Vijayakanth Pagadala[3], Aswini Panigrahi[1], Miloslav Sanda[1,4], Guowei Su[3], Radoslav Goldman[1]

[1]*Georgetown University, Clinical and Translational Glycoscience Research Center, Washington, DC 20057,* [2]*Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC,* [3]*Glycan Therapeutics, LLC, Chapel Hill, NC,* [4]*Max-Planck-Institut fur Herz- und Lungenforschung, Bad Nauheim, Germany,*

SULF1 and SULF2 are extracellular heparan 6-O-endosulfatases involved in the post-synthetic editing of heparan sulfate which regulates many important biological processes, including tumor growth and invasiveness. Despite their biological significance, activity of the SULF enzymes and their substrate specificity remain insufficiently characterized largely due to difficulties in the production and isolation of these highly modified proteins and due to the lack of well characterized synthetic substrates for probing their catalytic activity. We have generated stably transfected HEK293F and lentivirally-transduced HEK293T cells that allow stable production of milligram quantities of the SULF enzymes. We show that recombinant SULF1 and SULF2 proteins are N-glycosylated on up to 10 NXS/T sequons. We confirmed that SULF2, but not SULF1, is a proteoglycan carrying chondroitin sulfate chain attached to ^{583}SG motif. Proteomics analysis demonstrated that SULF enzymes produced in our overexpression system are a mixture of active and non-active forms of the SULF enzymes, due to partial conversion of Cys to formyl Gly in the active site. However, co-expression with formylglycine conversion enzymes SUMF1 resulted in a substantial increase in the proportion of the active form and in increased activity. To measure the 6-O desulfation activity of the SULF enzymes, we developed an assay using a p-nitrophenol labeled synthetic oligosaccharide GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP (2S2-6S4, Glycan Therapeutics) which is efficiently separated from the de-sulfated products by ion exchange HPLC chromatography and quantified by UV-absorbance. High enzyme:substrate ratios (up to 1:1,000,000) allow detection of low femtomoles of specific SULF enzyme activity in biological samples as complex as secretome of cancer cell lines. Our results show that the SULF activity depends on N-glycosylation and that the reaction buffer composition strongly affects the SULF enzymatic activity *in vitro*. The newly developed assay offers a simple, sensitive, and specific measurement of the heparan 6-O-endosulfatase activity that could open avenues to *in vivo* activity measurements and improve our understanding of the enzymatic editing of the sulfation of heparan.

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(P182) Nucleotide-sugar and glycan synthesis is limited by cellular reliance on glucose for energy production

Alfredo Reyes-Oliveras[1], ChongFeng Gao[1], Abigail Ellis[1], Ryan Sheldon[1], Brian Haab[1]

[1]*Van Andel Institute, 333 Bostwick Ave NE, Grand Rapids, MI, 49503,*

Background and hypothesis: The regulation of glycan synthesis is inherently tied to cellular metabolism because monosaccharides—particularly glucose—are converted to glycan precursors and are also used for energy generation. Cancer cells frequently increase their reliance on glucose for energy, but it is not clear whether such changes alter glycan production. We hypothesized that shifts in the use of glucose for energy alter the availability of glucose for conversion to nucleotide-sugar precursors and synthesis of extracellular glycans.
Methods: We developed a method to track isotope-labeled glucose carbons in the media on their journey through nucleotide-sugar precursors to mature membrane-bound glycans. We also developed a method for calibrating monosaccharide abundance thereby enabling comparisons between cell lines with widely varying metabolic characteristics or growth conditions. To validate the tracking of media-derived ^{13}C carbon towards intracellular and extracellular monosaccharides, we used cells with a CRISPR knockout of GFPT-1, the key enzyme in hexosamine biosynthesis, to confirm a specific decrease in labeling of the monosaccharides in the GFPT-1 pathway. We then compared patient-matched cell lines that have either high or low reliance on glucose as the primary energy source, PaTu8988T and PaTu8988S, respectively, and genetically modified AsPC-1 cells that have increased usage of glucose in aerobic glycolysis for energy production.
Results: The PaTu8988S cells imported less glucose than PaTu8988T cells and used less for energy (both by oxidative phosphorylation and glycolysis), but they used a greater amount of glucose to create nucleotide-sugar precursors. The higher routing of glucose to monosaccharide production led to a higher abundance of the corresponding extracellular glycans. Similarly, although the modified AsPC-1 cells greatly increased total glucose intake and glycolysis, they reduced the amount of glucose used for nucleotide-sugar precursors and extracellular glycans.
Conclusions: The commitment of glucose to energy production determines the glucose available for nucleotide-sugar precursor synthesis and glycan production. Cancer-cells that are adapted to glucose for energy correspondingly limit their extracellular glycosylation, suggesting that the extracellular glycosylation of cancer cells is fundamentally modulated by type of metabolic adaptation.

(P183) Reprogramming T cell Glycosylation: a novel immunotherapeutic strategy in Colorectal Cancer Treatment

Catarina M. Azevedo[1,2], Ângela Fernandes[2], Bingxian Xie[3,4], Rui M. Henrique[5,6], Mário Dinis-Ribeiro[7,8], Ricardo Marcos-Pinto[1,7,9], Greg M. Delgoffe[3,4], Salomé S. Pinho[1,2,10]

[1]*Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal,* [2]*i3S – Institute for Research and Innovation in Health, University of Porto, Porto, Portugal,* [3]*Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA,* [4]*Tumor Microenvironment Center, UPMC Hillman Cancer Center, Pittsburgh, PA, USA,* [5]*Department of Pathology and Cancer Biology and Epigenetics Group, IPO Porto Research Center (CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal,* [6]*Department of Pathology and Molecular Immunology, ICBAS, University of Porto, Porto, Portugal,* [7]*Centre for Research in Health Technologies and Information Systems (CINTESIS), University of Porto, Porto, Portugal* [8]*Department of Gastroenterology, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal,* [9]*Department of Gastroenterology, Porto Centre Hospital, Porto, Portugal,* [10]*Faculty of Medicine, University of Porto, Porto, Portugal,*

Colorectal cancer (CRC) is the second leading cause of cancer related death worldwide, remaining a serious public health problem in developed regions. Despite the clinical success of immunotherapy, only a minority of CRC patients benefit from this therapeutic modality, highlighting the urgent need for identifying novel mechanisms underlying cancer immunoregulation envisioning the improvement of CRC immunotherapies.

Changes in glycosylation are a hallmark of cancer. Glycans play a pivotal role in each pathophysiological step of malignant transformation (Pinho & Reis, Nature Reviews Cancer 2015) including in cancer immunoediting. Recent evidences from our group highlight the importance of glycans as relevant immune-checkpoints in cancer immunosurveillance and immunoediting. We showed that cancer cells-expressing complex branched *N*-glycans contribute to immune escape by promoting immunosuppressive networks through hampering IFNγ release (Silva MC & Fernandes Â. *et al.* Cancer Immunology Research, 2020). Moreover, and besides epithelial cells, glycans have been shown to play a key role in the regulation of T cell-mediated immune response (Dias A *et al.* PNAS 2018; Pereira M & Alves I *et al.* Frontiers Immunol 2018; Vicente, MM *et al.* Cell Mol Immunol 2023).

In this study, we investigated the dynamics of the glycosylation signature of different T cell populations along CRC progression, from pre-malignant lesions to full-blown CRC. Through *in silico* approaches (RNAseq analysis), the correlation between relevant glycogenes and immune genes was assessed. The results revealed the existence of a significant positive correlation between the

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expression of glycogenes involved in the branching *N*-glycosylation and the regulatory T cell response as well as the expression immune checkpoint molecules in the tumor microenvironment (TME). These results suggest a biological association of the expression of complex *N*-glycans in defining T cell properties in the TME associated with effective/ pro-inflammatory *versus* regulatory/ immunosuppressive programs. Additionally, using colorectal cancer mouse models (MC38-OVA-bearing mice), we observed that along tumor progression there is a gradual increase in the expression of complex *N*-glycans, that directly correlates with expression of immune checkpoint molecules on tumor infiltrating T cells (TILs). Furthermore, we demonstrated that the remodeling of those complex *N*-glycans from T cell surface, by *in vitro* glycoengineering had an impact in the regulation of T cell-mediated anti-tumor immune response. Taken together, these results suggest the key role of glycans in cancer immunosurveillance, supporting the importance of T cell glycosylation as a promising target for novel immunotherapeutic strategies.

(P184) Development and characterization of galectin-13-specific nanobodies

Camille Fuselier[1], Alyssa Dumoulin[1], Alex Paré[1], Rita Nehmé[1], Samy Ajarrag[1], Philippine Granger Joly de Boissel[1], David Chatenet[1], Nicolas Doucet[1], Yves St-Pierre[1]

[1]*INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC, Canada,*

Galectins (GALs) represent a family of evolutionarily conserved lectins that preferentially bind to glycoconjugate residues via their carbohydrate recognition domains (CRDs). Although GALs are known to play many roles during several physiological processes, their overexpression plays an essential role in the progression of several diseases, particularly cancer. Among galectins, galectin-13 (GAL-13) belongs to the subgroup of placental galectins. Their role in placental development and their use as a biomarker for gestational disorders has sparked the interest of many researchers in prenatal research. Initially identified in the syncytiotrophoblast of the placenta, there is increasing evidence that they also play a role in cancer progression. Indeed, cancer and trophoblast cells share several common features like invasive and angiogenic properties, while employing similar strategies to evade immune surveillance. Although it is logical to hypothesize that placental galectins play a role in cancer, the absence of specific inhibitors against GAL-13 has not yet enabled us to study their role in cancer and other diseases. Using sugar compounds as CRD inhibitors seems challenging since placental galectins such as GAL-13 and GAL-16 present the highest sequence homology among galectin family members. An alternative to this problem is to exploit the potential camelid antibodies (also called Nanobodies, Nbs) which have many advantages compared to conventional monoclonal antibodies. Indeed, their unique properties, such as their small size, high stability and high antigen-binding affinity, could allow Nbs to accelerate research on GAL-13 and serve as therapeutic agents. The objectives of our project were to develop Nbs against GAL-13 and an in vitro model to study its role in cancer by identifying Nbs with therapeutic potential. GAL-13-specific Nbs were generated by screening a synthetic camelid library with biotinylated full-length recombinant GAL-13 to find positive VHH clones. A hit-to-lead phase, comprising various biochemical and functional assays, was then used to identify lead Nbs. Our results showed that most, but not all, Nbs inhibited at different levels, the binding of GAL-13 to asialofetuin. A more effective inhibition was observed with selective combinations of Nbs, suggesting that several Nbs target distinct epitopes. We also determined the ability of the Nbs to target linear versus conformational epitopes and their sensitivity to mutations within GAL-13's glycan binding sites. The binding of the Nbs was specific for GAL-13, although, not surprisingly, some of them also bound GAL-16. In conclusion, we have generated GAL-13-specific Nbs with exciting properties, defining them as specific anti-GAL-13 inhibitors. As such, these Nbs are the first tools that could accelerate fundamental research on GAL-13 and the development of future clinical applications (imaging or therapy) for treating diseases where GAL-13 is overexpressed, notably in cancer.

(P185) AI-Driven glycoproteomic analysis identifies Nasopharyngeal Carcinoma biomarkers

Karina Islas Rios[1], Cheng Siang Lee[2], Ke En Tan[3], Alan Soo Beng Khoo[3], Chui Thean Low[3], Lu Ping Tan[3], Daniel Serie[4], Thin Thin Aye[2], Matthew P. Campbell[1]

[1]*InterVenn Biosciences Australia, 91-93 Victoria Parade, Fitzroy, Melbourne, Victoria, 3065, Australia,* [2]*InterVenn Biosciences, Technology Park Malaysia, Lebuhraya Puchong -Sg. Bukit Jalil. 57000, Kuala Lumpur, Malaysia,* [3]*Institute for Medical Research, Kuala Lumpur, Malaysia,* [4]*InterVenn Biosciences, 2 Tower Pl, 5th Floor, South San Francisco, CA 94080, USA,*

Nasopharyngeal carcinoma (NPC) remains mostly asymptomatic during early disease stages leading to late diagnosis and poor survival rates. Plasma Epstein-Barr virus (EBV) DNA and serology tests are advocated for early diagnosis and screening for NPC, but are of limited value because only a small proportion of patients with EBV develop NPC. Here, we present a MRM glycoproteomics pilot study (442 individuals) that identified 351 plasma glycopeptides differentially expressed between the healthy group and the NPC or NPC suspected groups. Out of these glycopeptides, 18 were selected to build a machine learning predictive model that differentiates NPC cases from healthy controls with an accuracy, sensitivity and specificity all above 89% in both training and test sets. The 18 selected glycopeptides include IgG, α2-macroglobulin, α1-antitrypsin and the complement pathway proteins Complement C2 and C4b-binding protein alpha chain. This preliminary investigation provides insight into

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the role of glycosylation in NPC progression, and suggests that more extensive studies of related glycoproteins and the plasma glycome may lead to better predictive models.

(P186) N-glycosylation by MGAT5 imposes a targetable constraint on T cell killing of pancreatic cancer cells

Erin Hollander[1], Rosemary Flock[2], Ben Stanger[3]

[1]Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Department of Biology, University of Pennsylvania, Philadelphia, PA, [3]Abramson Family Cancer Research Institute, Departments of Medicine and Gastroenterology, University of Pennsylvania, Philadelphia, PA,

Pancreatic ductal adenocarcinoma (PDAC) has a five-year survival rate of less than 12%, a dismal rate attributed to a difficulty in early detection and a lack of effective treatments. One potential target is the glycosyltransferase MGAT5, which catalyzes the formation of β1,6-N-acetylglucosamine branched glycans. Overexpression of MGAT5 has been implicated in tumor growth and metastasis in multiple cancers.

Using a panel of clonal cell lines that recapitulate the immune heterogeneity of PDAC, we found that loss of MGAT5-mediated N-glycans allows for robust abrogation of tumor growth. By contrast, MGAT5 loss has no impact on tumor cell growth *in vitro*. This phenotype appears to be dependent upon the presence of T cells and dendritic cells, with NK cells playing a role in early tumor clearance. Immune phenotyping of the tumor microenvironment of control and MGAT KO tumors *in vivo* revealed an increased anti-tumor immune microenvironment, with significantly increased tumor-infiltrating T cells and NK cells and decreased gMDSCs.

To delineate the mechanism underlying this robust tumor clearance, OT-I T cells targeting the strong antigen ovalbumin were cultured with both control and MGAT5 KO PDAC cells engineered to express ovalbumin. The KO tumor cells had a higher rate of killing by T cells in the co-culture. Upon probing different methods of killing by T cells, MGAT5 KO cells were found to be exceptionally sensitive to TNFα-mediated cell death even at very low concentrations. Knockout of MGAT5 in the non-PDAC lines MC38 (colon adenocarcinoma), LLC (Lewis lung carcinoma), and B16 (melanoma) found similar increased sensitivity to TNFα-mediated cell death in the knockout lines, suggesting MGAT5 glycans may be responsible for abrogating the TNFα apoptotic response in multiple cancers.

These results are consistent with a model in which loss of MGAT5-mediated N-glycans increases the sensitivity of tumors to T cell killing through the TNFα pathway, allowing for the formation of a durable immune response. Finally, MGAT5 knockout tumors treated with immune checkpoint blockade had significantly decreased tumor size and increased survival over controls, suggesting MGAT5 has potential as a novel target for pancreatic cancer.

(P187) *In vitro* and *in silico* analyses of oligo/polysialoglycoconjugates in cancer cells

Masaya Hane[1,2,3], Ken Kitajima[1,2,3], Chihiro Sato[1,2,3]

[1]Institute for Glyco-core Research, Nagoya University, [2]Graduate School of Bioagricultural Sciences, Nagoya University, [3]Bioscience and Biotechnology Center, Nagoya University,

Including vertebrates, almost all deuterostomes contain sialic acids (Sias) at the non-reducing termini of their glycans. Therefore, sialoglycoconjugates exist in the outermost part of cells and play important roles in cellular functions. Alterations in cell-surface glycosylation are well known in several diseases as previously reported. In cancer cells, several glyco-epitopes on the cell surface, especially sialyl glyco-epitopes such as sialyl Lewis A (sLe^a, CA19-9) and sialyl Lewis X (sLe^X, SLX), are prominent, and generally used as biomarkers. Sias are categorized into three major groups based on the modification of the C-5 position: *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (Kdn). In vertebrates, Neu5Ac and Neu5Gc are mainly expressed. However, human cells, which lack functional CMP-Neu5Ac hydroxylase (CMAH), can only synthesize Neu5Ac, and exclusively express Neu5Ac-containing glycoproteins and glycolipids (Neu5Ac-glycoconjugates) on the cell surface. Nevertheless, the occurrence of Neu5Gc- and Kdn-glycoconjugates in human cancer cells has been reported, thus suggesting that Neu5Gc- or Kdn-glycan epitopes could be a biomarker for some cancers. Sias are sometimes linked to each other to form oligo/polySia structure. Since they are unique and rarely expressed in normal cells, oligo/polySia structures may also alter in a disease-dependent manner. Thus, we are hypothesizing that oligo/polySia structures serve as promising novel biomarkers and targets for therapies. For their application to the diagnosis and treatment of some cancers, a precise understanding of the oligo/polySia structures in cancer cells is necessary.

To demonstrate the hypothesis, we focused on oligo/polySia structures containing minor Sia components (Neu5Gc/Kdn) in human. We performed flow cytometric analysis with several anti-sialoglycoconjugate antibodies using several kinds of cancer cell lines. In addition, gene expression datasets were obtained from each cell lines, from which genes for glycosyltransferases and the corresponding substrate proteins were extracted. We found that, based on these datasets, the glycan structures and their sialylation status could be predicted. Interestingly, our results also showed that sialylation patterns are unique to each cancer cell

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line. Thus, we suggest promising combinations of antibody and cancer cell can be selected for major glycan prediction. However, further biochemical analysis is required for the precise prediction including minor glycans.

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(P188) Using glycan targeting drugs to block prostate cancer bone metastasis

Kirsty Hodgson[1], Emily Archer Goode[1], Kayla Bastian[1], Matthew Fisher[2], Rebecca Garnham[1], Margarita Orozco-Moreno[1], Emma Scott[1], Rashi Krishna[1], Richard R. Drake[3], David J. Elliott[1], Ning Wang[2], Jennifer Munkley[1]

[1]Newcastle University Centre for Cancer, Newcastle University Institute of Biosciences, Newcastle, NE1 3BZ, UK, [2]The Mellanby Centre for Musculoskeletal Research, Division of Clinical Medicine, The University of Sheffield, Sheffield, UK, [3]Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina,

Bone metastasis is a common, debilitating, and incurable consequence of advanced prostate cancer. New treatments for bone metastasis are urgently needed and could significantly impact patient quality of life and survival times. Why prostate cancer cells metastasise to bone and by what mechanisms are big unanswered questions. Our data suggest that glycans provide a key part of the answer and could be targeted to block prostate cancer spreading to bone. We have discovered that sialylated and core fucosylated *N*-glycans are upregulated in aggressive prostate cancer tissue and this can promote prostate cancer bone metastasis. We have generated proof-of-principle data to show these glycans can be targeted therapeutically. To build on this research and generate the data we need to translate these treatments into the clinic, we are utilising innovative mouse models to investigate the importance of sialylation and core fucosylation in prostate cancer bone metastasis and testing if glycan-targeting drugs can be developed into new therapies to block prostate cancer spreading to bone. Moving forward, we envisage combining glycan-targeting drugs with existing therapies for prostate cancer to develop game-changing treatment strategies for advanced prostate cancer. This work was funded by Prostate Cancer Research and the Mark Foundation for Cancer Research (grant reference 6961).

(P189) Galectin inhibitors: A new generation of single-domain antibodies to target both intracellular and extracellular galectins for cancer treatment

Rita Nehmé[1], Marlène Fortier[1], Myriam Létourneau[1], Philippine Granger Joly de Boissel[1], Alyssa Dumoulin[1], Camille Fuselier[1], David Chatenet[1], Nicolas Doucet[1], Yves St-Pierre[1]

[1]INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, QC, Canada,

Galectins are a family of evolutionarily conserved lectins known for their preference for β-galactoside-containing glycoconjugates. Based on their carbohydrate-recognition domains (CRDs), the 12 members of the human galectin family are classified as prototypic, tandem-repeat type and chimeric-type. Galectins perform various homeostatic functions both inside and outside the cells. However, abnormally high levels of galectins can be expressed by cancer cells. The conventional wisdom has been that galectins play a central role in cancer by creating local and systemic immunosuppression, enabling cancer cells to escape the host's immune defense. While significant attention has been directed towards extracellular galectins and their glycan-binding activity, galectins are also well-known for their ability to promote cancer progression intracellularly. Due to these factors, there has been an increased interest in using galectins as therapeutic targets. Despite considerable efforts toward the development of specific galectin inhibitors, limited success has been met until now. This is due primarily to the lack of highly specific, high-affinity galectin inhibitors and the limited understanding of each galectin's role in cancer progression. Here, we present the results of several years of research that led to the development of a diverse panel of functional galectin-specific nanobodies (Nbs) and their genetically engineered minibody forms. GAL-7-specific Nbs were generated by screening a synthetic camelid library with biotinylated full-length recombinant galectins. Following three rounds of selection, VHH clones were randomly selected and tested in a non-absorbed phage ELISA assay using avidin plates and biotinylated galectins. Positive clones were selected based on their amino acid sequences, and their cDNA was cloned into the pHEN2 expression vector. In certain cases, the cDNA encoding the Nbs was inserted into pFUSE vectors to generate minibodies with a human IgG1-Fc fragment or was used to generate galectin-specific intrabodies, including PROTACs.Overall, we successfully generated more than 40 Nbs and minibodies targeting galectin-1, -2, -7, -9, and -13-16. We have also generated vectors encoding ALFA-tagged intrabodies to target intracellular pools of galectins and intrabodies fused to the von Hippel–Lindau (VHL) protein to redirect intracellular galectins to the ubiquitination machinery. Hit-to-lead phases led to the identification of high-affinity, highly specific and functional Nbs capable of inhibiting galectin-induced apoptosis. Lead Nbs for galectin-1 and galectin-7 were also tested in vivo for their ability to target galectin-positive mammary tumors using PET/CT imaging. In addition to providing a novel set of research tools to

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study galectins, our work opens the way to new treatments for different types of cancer, especially for hard-to-treat cancers where galectins have been shown to play an important role in tumor progression.

(P190) 3-O-sulfated heparan sulfate-induced EGFR signaling activation drives acquisition of castration resistance in prostate cancer

Hayato Ota[1], Hayato Ota[1], Hirokazu Sato[1], Shuji Mizumoto[2], Ken Wakai[3], Kei Yoneda[3], Kazuo Yamamoto[3], Hayao Nakanishi[4], Jun-Ichiro Ikeda[3], Shinichi Sakamoto[3], Tomohiko Ichikawa[3], Shuhei Yamada[2], Satoru Takahashi[5], Yuzuru Ikehara[3], Shoko Nishihara[1,6]

[1]*Graduate School of Engineering, Soka University,* [2]*Faculty of Pharmacy, Meijo University,* [3]*Graduate School of Medicine, Chiba University,* [4]*Aichi Cancer Center Aichi Hospital,* [5]*Graduate School of Medical Sciences, Nagoya City University,* [6]*Glycan & Life System Integration Center (GaLSIC), Soka University,*

Prostate cancer is one of the most common cancers that occur in male. Usually, prostate cancer proliferates through androgen signaling. Androgen deprivation therapy (ADT) is given to suppress prostate cancer growth. However, some prostate cancer cells are resistant to ADT and grow hormone-independently as castration resistant prostate cancer (CRPC). Reliable treatment for CRPC has not been established. Sulfated glycosaminoglycans promote ligand binding to receptors as co-receptors, but their role in CRPC remains unknown. Using the human prostate cancer cell line C4-2, which can proliferate in hormone-dependent and hormone-independent conditions, we found that the expression of 3-*O*-sulfated heparan sulfate (3-OS HS) synthesized by HS 3-*O*-sulfotransferase 1 (HS3ST1) was significantly upregulated in hormone-independent growth condition. Knockdown (KD) of *HS3ST1* gene in C4-2 cells suppressed hormone-independent growth. Furthermore, epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF) -activated EGFR–ERK1/2 signaling was suppressed in *HS3ST1* KD C4-2 cells. Importantly, we revealed that EGFR activation in C4-2 under hormone depleted condition was facilitated not only by HB-EGF, which possesses a heparin-binding domain, but also by EGF lacking this domain, through 3-OS HS. EGF binding to the living cell surface was inhibited in *HS3ST1* KD C4-2 cells. In addition, we investigated the possibility of targeting EGFR signaling in the treatment of prostate tumors. Gefitinib, an EGFR inhibitor, significantly suppressed C4-2 cell proliferation and growth of a xenografted C4-2 tumor in castrated mouse. Collectively, our study has revealed an acquisition mechanism of castration resistance in prostate cancer via EGFR activation mediated by 3-OS HS. (Reference: Ota *et al. Sci Rep* 13:11618, 2023)

(P191) Glycomic Profiles of Breast Cancer Tissue Through the Lens of HER2 and ER Expression

Clay Elliott[1], Avery Funkhouser[1], Julie Martin[2], Jeffery Edenfield[2], Larry Gluck[2], Connie Arthur[3], Anna Blenda[1,2]

[1]*University of South Carolina School of Medicine Greenville,* [2]*Prisma Health Cancer Institute,* [3]*Harvard Medical School,*

Abnormal glycan expression caused by genetic mutations or cellular stressors is a known characteristic of disease states like cancer. Thus, finding variations of glycan expression in cancer tissue might be useful for diagnosis and staging. Earlier research has shown that breast cancer tumor tissue has elevated high mannose, complex N-linked, and hybrid glycan expression when compared to the surrounding unaffected tissue. We have shown that specific high mannose glycans, H5N2, H6N2, H7N2, H8N2 and H9N2, were elevated in stage I breast cancer tissue when compared to surrounding non-cancerous tissue ($p<0.05$). This data provides promising information that could be used for early breast cancer detection and tumor demarcation. In many breast cancers, HER2 or ER activation is a driving factor that promotes downstream gene expression that stimulates unregulated cellular growth. Because elevated glycan expression in cancer is attributed to unregulated cellular growth, we wanted to find if HER2 or ER expression correlated with elevated glycan expression. Breast tissue tumor samples were collected from 39 breast cancer patients at the time of surgery. Tissue samples were later obtained for glycomic analysis from the Prisma Health Cancer Institute Biorepository with their clinical records including HER2 and ER status. Glycomic profiles of the tissue samples were obtained using MALDI-TOF Mass Spectroscopy by Emory Glycomics and Molecular Interactions Core. Statistical analysis of the breast cancer samples revealed a total of 10 unique glycans that were abnormally elevated in expression in HER2 positive breast cancer tissue when compared to HER2 negative breast cancer tissue ($p<0.05$). More specifically, high mannose glycans H5N2, H6N2, H7N2 and H8N2 were upregulated in HER2 positive breast cancer tissues ($p<0.05$). A similar elevation of high mannose glycan expression was seen in ER negative breast cancer tissue ($p<0.05$). This data expands previous research done in the field of breast cancer glycomic profiling. High mannose glycans were shown to be elevated in many cancers making them a promising glycomic marker for cancer identification and diagnosis. Our findings suggest that the molecular profile of breast cancer influences glycan expression in cancer tissue. Further research into the effect that breast cancers molecular profiles have on glycan expression could help us better understand how these cancers progress and metastasize.

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(P192) CD38 Glycosylation Determines Antibody Recognition in Myeloid Leukemia Cell Lines

Agata Steenackers[1], Katelyn E. Rosenbalm[1], Natalia Weich[1], Karin M. Hoffmeister[1,2]

[1]*Translational Glycomics Center, Versiti Blood Research Institute, Milwaukee, WI, USA,* [2]*Departments of Medicine and Biochemistry, Medical College of Wisconsin, WI, USA,*

Acute leukemia is a hematologic malignancy with an unsatisfactory prognosis, particularly in relapsed or refractory acute myeloid leukemia (AML), underscoring the need for novel therapeutic approaches. Recent advancements include targeted and immune therapies, focusing on surface antigen CD38 due to its high expression across various hematologic malignancies. CD38-targeted therapies, such as monoclonal antibodies (mAbs), bispecific antibodies, and CAR-T cell therapy, have demonstrated promising efficacy with manageable toxicity. Some patients develop resistance despite sustained CD38 expression. With 4 putative N-glycosylation sites, glycan expression patterns on CD38 may alter immune response and facilitate treatment escape. The impact of CD38 glycan expression on target recognition, effector function, and its composition on malignant and therapy-resistant leukemia cells is unclear. We hypothesized that variations in CD38 glycosylation patterns depend on the leukemia type and stage, which could affect therapeutic approaches. To test this hypothesis, we examined the relationship between CD38 expression levels, glycosylation patterns, and their combined effect on antibody binding across several leukemia cell lines representing different types of leukemia and disease stages (HL-60, Acute Promyelocytic Leukemia; MV4-11, Myelomonocytic Leukemia; LAMA-84, Chronic Myeloid Leukemia at Blast Crisis). By flow cytometry and immunoblot analysis we identified substantial heterogeneity in CD38 surface antigen expression levels and distinct CD38 glycoform profiles between cell lines.Furthermore, we investigated the effects of all-trans retinoic acid (ATRA) on CD38 expression in different leukemia cell lines. ATRA enhanced the lytic capacity of CD38 targeted therapies by increasing CD38 levels on target cells. Our results showed a varied response to ATRA treatment across leukemia cell lines. HL-60 and MV4-11 cell lines, which had low baseline CD38 expression, exhibited a significant induction of CD38 expression after ATRA treatment. In contrast, the LAMA-84 cell line did not demonstrate significant change in CD38 expression following ATRA treatment. Additionally, we assessed glycosylation alterations in these cell lines using lectin arrays on cell extracts and mass spectrometry of N-glycans. While the HL-60 cell line exhibited a significant increase in CD38 expression post-ATRA treatment, the glycosylation patterns remained mostly stable. In contrast, the LAMA-84 cell line, which displayed no change in CD38 expression after ATRA treatment, showed significant alterations in its glycosylation patterns. Importantly, changes in glycosylation in the LAMA-84 cell line had a substantial impact on the binding affinity of anti-CD38 antibodies.Our findings underline the complexity of the interplay between CD38 expression and glycosylation in leukemia, highlighting a critical area for further investigation to optimize therapeutic strategies.

(P193) Identification of Polysialylated Proteins in Breast Cancer and Evaluation of their Role in Metastasis

Sepideh Soukhtehzari[1], Jacob Melamed[1], Nikki Salmond[1], Sumreen Javed[1], Karla C. Williams[1]

[1]*Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada,*

Posttranslational modification of proteins through cellular glycosylation is essential in the regulation of many different physiological processes. Altered glycosylation is also a hallmark of cancer and has been implicated in tumor progression and metastasis. A striking example of aberrant glycosylation in cancer is the noticeable change in expression of polysialic acid (polySia). PolySia is synthesized by two Golgi-resident sialyltransferases, ST8Sia2 and ST8Sia4 and in a healthy adult expression is restricted to a number of cell types and proteins. We previously identified polySia expression in breast cancer patient tumors and identified ST8Sia4 as the polysialyltransferase expressed in these tissues. As our work identified a significant correlation between ST8Sia4 and polySia expression and poor patient outcomes, here we aimed to study the role of ST8Sia4 and polysialylated proteins in breast cancer metastasis. To identify polySia carriers, we immunoprecipitated polysialylated proteins from MCF7 cell lysate and analysed SDS-PAGE extracted bands by Mass spectrometry. We validated two candidate proteins (QSOX2 and GLG1) using western blot and immunoprecipitation (+/-EndoN was used to selectively cleave polySia chains), and conducted immunocytochemistry for colocalization studies. Stable knockdown and knockout cell lines for ST8Sia4, GLG1, and QSOX2 were generated and assessed tumor cell proliferation, extravasation and metastasis using chick embryo chorioallantoic membrane (CAM) model. PolySia, and polysialylated proteins, expression was evaluated on metastatic brain patient-derived xenografts (PDX) by fluorescence-based immunohistochemistry. Here, we identified and validated QSOX2 protein as a novel polySia carrier in MCF7 cells. In addition, we found that GLG1 (a known polysialylated protein) is also polysialylated in MCF7 cells. In Vivo functional studies showed that knockout, or knockdown, of ST8sia4, QSOX2, or GLG1 significantly reduced tumor cell proliferation and metastatic brain tumor burden compared to control. While QSOX2 and GLG1 knockout cell lines demonstrated significant reductions in tumor cell extravasation, no significant difference was identified in ST8Sia4 knockdown cell lines. Immunostained PDX tissue demonstrated marked expression of polySia, ST8Sia4, and polysialylated proteins.Our

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finding suggests that polysialylated QSOX2 and GLG1 have important roles in breast cancer cell proliferation and metastasis. As we identified expression in metastatic brain tumors from breast cancer patients, understanding how these polysialylated proteins promote tumor metastasis will be an important next step.

(P194) Mapping miRNA Governing Sialylation Reveals Cancer-Associated Upregulatory Networks

Faezeh Jame Chenarboo[1], Joseph Reyes[1], Nicholas Twells[1], Hoi Hei Hg[1], Dawn Macdonald[1], Lara Mahal[1]

[1]*Department of Chemistry, University of Alberta, Edmonton, AB T6G 2G2,*

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microRNAs (miRNAs, miRs) are small, non-coding RNA that are widely considered as posttranscriptional repressors, binding to the 3'-UTR of mRNA within RISC complex and causing mRNA destabilization and/or loss of translation. Using our recently developed high-throughput assay (miRFluR) (1), we have comprehensively mapped the miRNA regulatory landscape of sialyltransferase enzymes (ST6GAL1, ST6GAL2) (2). Our recently published work revealed, contrary to expectations, that miRNA can both upregulate and downregulate protein expression through direct interactions. Indeed, for some enzymes, upregulation is the predominant mode of action. The miRNA binding proteins AGO2 and FXR1 were required for upregulation. Our results upend common assumptions surrounding miRNA, arguing that upregulation by these non-coding RNA is common. Our work also provides another potential pathway to explain the dysregulation observed in cancer and other disease states through dysfunction of miRNA regulation. In recent work, we have identified alpha-2,3-sialylation by ST3GAL1 and ST3GAL2 as a molecular driver of CD98hc (encoded by slc3a2) glycoprotein in melanoma. Mapping the miRNA landscape for both the sialyltransferases (ST3GAL1, ST3GAL2) and their glycoprotein target (CD98), we have found that these targets are synergistically regulated by upregulatory miRNAs involved in melanoma pathogenesis. The co-upregulation of glycans and their targets by miRNA regulatory networks adds a new twist to the impact miRNA have on glycosylation. 1. Thu, C. T.; Chung, J. Y.; Dhawan, D.; Vaiana, C. A.; Mahal, L. K., High-Throughput miRFluR Platform Identifies miRNA Regulating B3GLCT That Predict Peters' Plus Syndrome Phenotype, Supporting the miRNA Proxy Hypothesis. ACS Chem Biol 2021, 16 (10), 1900–1907. 2. Jame-Chenarboo, F., Hoi Hei Ng, Dawn MacDonald, Lara K. Mahal, ACS Cent. Sci., 2022, 8 (11),1527–1536.

(P195) ST6Gal1-Mediated α2,6 sialylation Modulates Glioblastoma Metabolism

Joelle Saad[1], G.C. Sajina[1], Travis Nemkov[2], Kaysaw Tuy[1], Sam Gary[1], Victor Darley-Usmar[2], Susan Bellis[1], Anita Hjelmeland[1]

[1]*Department of Cell, Development and integrative Biology, University of Alabama at Birmingham, Birmingham, AL, USA,* [2]*Department of Biochemistry and Molecular Genetics, University of Colorado Denver, Denver, CO, USA,* [3]*Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA,*

Glioblastoma (GBM) is the most frequent and highly malignant primary brain tumor that predominantly occurs in individuals over 50 years of age. Despite the use of the current standard of care, involving maximal surgical resection followed by radiation and chemotherapy, the median survival is only around 15 months. This poor patient prognosis is partly driven by the therapeutically resistant and metabolically plastic subpopulation of brain tumor initiating cells (BTICs). Alterations in metabolic profiles represent one mechanism underlying this resistance. Hence, elucidating regulators of BTICs metabolism provides potential targets for therapeutic intervention, and glycosylation is one such regulator. Glycosylation has been shown to modulate the structure and function of cell surface and secreted proteins leading to maintenance of cellular homeostasis in the presence of extracellular environment changes. Beta-galactoside α2,6-sialyltransferase 1 (ST6Gal1) is a glycosyltransferase that adds α2,6 linked sialic acid to N-glycans on glycoproteins. ST6Gal1 is a subject of intense research, and our lab has identified ST6Gal1 to be upregulated in GBM, specifically in the BTICs population, and that it regulates a subset of cell-surface proteins. However, impacts of ST6Gal1 on GBM metabolism remain unexplored. A pressing need therefore exists to study the effect sialylation has on glycoproteins, including those involved in regulating metabolism. To decipher the role of α2,6 sialylation in regulating GBM metabolism, we have acquired metabolomics profile from GBM patient derived xenografts with ST6Gal1 modulation. Our global metabolomic profile data suggest altered metabolic pathways with the loss of ST6Gal1, namely: glycolysis, fatty acid oxidation, nitrogen metabolism, and oxidative stress. This is the first report linking changes in sialyltransferase to global metabolome effect in GBM. Supporting these data, we also find that α2,6 sialylation high GBM cells have elevated glycolytic capacity and increased glucose uptake. As it has been established that α2,6 sialylation by ST6Gal1 can alter conformation, clustering, and retention of glycoproteins, we have identified potential downstream targets of ST6Gal1 that regulate metabolism. Using high resolution imaging and flow cytometry, we are studying the cell-surface dynamics and function of these glycoproteins after ST6Gal1 modulation. Taken together, our work will elucidate the effect of ST6Gal1-mediated α2,6 sialylation on metabolism and uncover novel GBM reprogramming pathways and potential therapeutic targets.

(P197) Identification of TAG-72 carriers and glycoproteomic mapping of the epitope

Egan L. Peltan[1,4], David S. Roberts[2,5], Nicholas M. Riley[3], Carolyn R. Bertozzi[2,4,5,*]

[1]Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA, USA 94305, [2]Department of Chemistry, Stanford University, Stanford, CA, USA 94305, [3]Department of Chemistry, University of Washington, Seattle, WA, USA 98195, [4]Sarafan ChEM-H, Stanford University, Stanford, CA, USA 94305, [5]Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA,

*Corresponding Author

The hybridoma technique enabled the identification of cancer-specific monoclonal antibodies for targeted cancer therapy using tumor immunization. Identification of the targets of tumor specific antibodies revealed tumor specific and tumor associated antigens. Identified in 1984, TAG-72 is a tumor-associated antigen defined by two monoclonal antibodies B72.3 and CC49. The Fabs of anti-TAG-72 mAbs have been used to direct passive immune therapy, radiotherapy, antibody-drug conjugates, CAR-T cells, and most recently by Tagworks Pharmaceuticals click-to-release ADCs. Yet, the identity of TAG-72 carriers, and the nature of the native epitope, remain unknown. Using Immunoprecipitation-Mass Spectrometry and CRISPR-Cas9, we identified MUC2 as a major TAG-72 carrier in the LS174T model of colorectal cancer. Further, we show that the TAG-72 epitope emerges from the loss of T-synthase activity and create TAG-72+ CD43 in K562 following T-synthase loss. We confirm the sialic acid requirement of the TAG-72 epitope and observe notable sensitivity to select mucinases and hydroxide. Using a glycoproteomic approach, we demonstrate that anti-TAG-72 antibodies recognize distinct patterns of Sialyl-Tn antigens hosted on a subset of disordered S/T-rich regions on the N-terminal mucin-like domain of CD43. Further, we show that anti-TAG-72 antibodies can inhibit the engagement of immune receptors by STn bearing proteins, including a novel sialic-acid dependent interaction between CD43/CD43-STn and an immune receptor.

(P198) Design of a mucin-selective protease for targeted degradation of cancer-associated mucins

Gabrielle Tender[1], Kayvon Pedram[1], D. Judy Shon[1], Natalia R. Mantuano[2,3], Jason J. Northey[4], Kevin J. Metcalf[4], Valerie M. Weaver[4,5], Heinz Läubli[2,3], Carolyn R. Bertozzi[1,6]

[1]Stanford University, Department of Chemistry and Sarafan ChEM-H, Stanford, CA 94305, USA, [2]Cancer Immunotherapy Laboratory, Department of Biomedicine, University of Basel, Basel, BS 4031, Switzerland, [3]Division of Oncology, Department of Theragnostics, University Hospital, Basel, BS 4031, Switzerland, [4]Center for Bioengineering and Tissue Regeneration, Department of Surgery, University of California, San Francisco (UCSF), San Francisco, CA 94143, USA, [5]Departments of Radiation Oncology and Bioengineering and Therapeutic Sciences, Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, and Helen Diller Comprehensive Cancer Center, UCSF, San Francisco, CA 94143, USA, [6]Howard Hughes Medical Institute, Stanford, CA 94305, USA,

Mucins are densely O-glycosylated glycoproteins that have been shown both clinically and experimentally to play important roles in cancer progression. Their upregulation and altered glycosylation status are some of the most common cancer-associated changes, and they are leading cancer prognostic and diagnostic markers. Prior mechanistic work has demonstrated that increased mucin expression on cancer cells enables proliferation at metastatic niches by promoting survival under low adhesion settings and reduces immune cell killing of tumor cells by boosting immune-inhibitory signals.

Decades of functional, genetic, and preclinical data support depletion of cancer-associated mucins as a strategy to reverse tumor progression. However, there are so far no therapeutics that reverse the pleiotropic functions of mucins. This is because mucins are a protein class comprised of repeated peptide domains highly modified with variable, branching, non-genetically encoded, and biosynthetically complex glycans, leading to an extended "bottle brush" conformation, all of which combine to promote cancer progression. These properties complicate attempts to drug them with small molecule inhibition or antibody blockade.

Here we present a novel way to deplete cancer-associated mucins through engineering and targeting of a mucin-selective protease ("mucinase") to cancer cells. Using structure-guided engineering, we reduced the activity of the mucinase to avoid off-tumor mucin cleavage. When the engineered enzyme is concentrated on the cancer cells via a targeting agent, the high local molarity of substrate drives on-tumor mucin cleavage.

We fused an anti-HER2 nanobody to the mutated mucinase which degraded mucins on only targeted (HER2+) cells in mixed cell cleavage assays. We validated the generalizability of this approach by targeting the mucinase to a wide variety of other cell surface markers. In mixed cell models of biophysical and immunological mucin-dependent tumor-progressive pathways, the nanobody-mucinase conjugate selectively killed HER2+ cells, indicating reversal of these pathways on only the targeted cells.

To probe efficacy *in vivo*, we used previously validated mouse models of breast cancer progression. In two models, the nanobody-mucinase conjugate, and not the inactive or non-targeting nanobody-mucinase controls, blunted primary tumor burden and metastatic outgrowth. We also observed targeted degradation of mucins on the tumor cells *in vivo*.

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In addition to outlining a first in class approach to degrade previously undruggable cancer-associated mucins, this work more broadly expands the targeted protein degradation space by bringing the engineered protease directly to the cells of interest. In addition, this strategy establishes a blueprint for the development of biologics that degrade specific protein glycoforms on target cells.

(P199) ST6GAL1-mediated α2,6 Sialylation in Brain Tumor Initiating Cells

Anita Hjelmeland[1]

[1]*Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA.,*

Glioblastoma is a highly aggressive malignant, primary brain tumor with a median life expectancy of approximately 15 months past diagnosis. One of the many reasons that glioblastoma is difficult to treat involves a subset of tumor cells with characteristics of neural stem cells called brain tumor initiating cells (BTICs) or glioblastoma stem cells. Through improved understanding of BTIC glycobiology, our laboratory seeks to better understand and treat glioblastoma. We recently published (GC et. al, *JCI Insight*, 2022) that an enzyme which adds α2,6 linked sialic acids to *N*-glycosylated proteins, called beta-galactoside α2,6-sialyltransferase or ST6GAL1, is elevated in BTICs. We found that glioblastoma α2,6 sialylation low cells or BTICs with *ST6GAL1* knockdown have decreased growth *in vitro* as well as decreased neurosphere formation, an indicator of BTIC self-renewal. A sialyltransferase inhibitor preferentially decreased the growth of BTICs, suggesting the future potential of ST6GAL1 inhibitors against glioblastoma. Mice orthotopically injected with α2,6 sialylation low glioblastoma cells or *ST6GAL1* knockdown BTICs have increased survival in comparison to mice injected with α2,6 sialylation high cells or non-targeting control BTICs, respectively. Mechanistically, we determined that *ST6GAL1* sialylates *PDGFRB*, which is known to be elevated in BTICs and regulate their tumorigenic potential. We continue to determine the biological and molecular roles of ST6GAL1 in glioblastoma and have recently defined a novel role for ST6GAL1-mediated α2,6 sialylation in the regulation of BTIC metabolic plasticity. Considering that ST6GAL1 can be active in the Golgi, as a secreted form in the extracellular space, or in exosomes, there are many mechanisms through which ST6GAL1 could impact both glioblastoma cells and the tumor microenvironment. Thus, we are only beginning to understand how brain tumors are regulated by ST6GAL1-mediated α2,6 sialylation. This work has been supported by the National Institutes of Health (R01NS127424; R03NS125506).

(P200) Examining Sialylation Impacts on the Glioblastoma Microenvironment

Anitha Parthiban[1], Anita Hjelmeland[1]

[1]*Department of Cell, Development and integrative Biology, University of Alabama at Birmingham,*

Glioblastoma is the most common primary malignant brain tumor with a median survival of 15 months, even with maximum surgical resection and radio- and chemotherapy. Glioblastoma treatment is complicated by the presence of highly tumorigenic glioblastoma cells with properties of neural stem cells, called brain tumor initiating cells (BTICs), which are resistant to standard of care and repopulate the tumor. We recently published that BTICs express high levels of the sialyltransferase ST6Gal1 (galactoside α2,6-sialyltransferase 1) which adds sialic acid to the terminal galactose of N-glycoproteins. We found a protumorigenic role for ST6Gal1-mediated α2,6-sialylation in BTICs. Beyond this direct role in glioblastoma cells, ST6GAL1 from BTICs could also act on non-neoplastic cells in the glioblastoma microenvironment. For example, glioblastoma associated macrophages occupy up to 50% of the bulk tumor, and recent published reports suggest an important role for sialylation and ST6GAL1 in the regulation of myeloid cells and immunosuppressive macrophages. Glioblastoma associated macrophages contribute to an immunosuppressive environment and are also implicated in promoting invasion as well as therapeutic resistance. Thus, we are exploring whether ST6GAL1-mediated sialylation of glioblastoma associated macrophages could contribute to multiple facets of tumor biology.

(P201) Toward Finding A Cure for NGLY1 Deficiency

Tadashi Suzuki[1,2]

[1]*Glycometabolic Biochemistry Laboratory, RIKEN Cluster for Pioneering Research (CPR), Saitama, Japan,* [2]*Takeda-CiRA Joint Program (T-CiRA), Kanagawa, Japan,*

The cytoplasmic peptide:N-glycanase (PNGase; NGLY1 in humans) is a deglycosylating enzyme widely conserved in eukaryotes (1). This enzyme is involved in the degradation of misfolded *N*-glycosylated proteins destined for proteasomal degradation in the cytosol, a process called endoplasmic reticulum-associated degradation (ERAD). The discovery of NGLY1 deficiency, a

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human genetic disorder bearing mutations in the *NGLY1* gene, has led to rapid research progress on this protein (2). We have been analyzing various *Ngly1*-KO animals (mice/rats/flies), and have identified genetic factor(s) greatly affecting the phenotypic consequences of these animals (3, 4). It is particularly worth noting that additional KO of *Fbs2*, a glycan-recognizing E3 ubiquitin ligase subunit, can rescue the embryonic-lethal phenotype of *Ngly1*-KO mice, and the *Ngly1 Fbs2* double-KO mice exhibited no obvious defect on their motor functions (4). These genes can thus be regarded as a promising drug target for NGLY1 deficiency. Moreover, recent evidence also suggested that AAV9-based gene therapy could be a viable therapeutic option for NGLY1 deficiency (5). In this lecture, I will summarize the most recent progress of our efforts to find a cure for NGLY1 deficiency.

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(P202) N-Glycans on Proteins: Homogeneous glycoprotein synthesis and their interesting functions

Yasuhiro Kajihara[1]

[1]*Department of Chemistry, Osaka University,*

Asparagine-linked oligosaccharides (N-glycan) of protein play important roles in many biological events. In order to evaluate the function of N-glycans depending on glycosylation positions and glycosylation number, we have synthesized homogeneous glycoproteins. To synthesize glycoproteins, we prepared homogeneous N-glycan from egg yolk over a gram scale. Using these glycans, we synthesized fluoromethyloxycarbonyl (Fmoc)-Asn-oligosaccharide or its tert-butylcarbonyl (t-Boc) forms for solid phase peptide synthesis (SPPS). Glycopeptides and peptides thus prepared were then coupled by native chemical ligation to yield a full-length target glycosyl polypeptide. Subsequent in vitro folding protocol successfully generated a folded glycoprotein. Recently, we further developed a synthesis method utilizing glycosyl asparagine thioacid. This thioacid functional group serves to couple the N and C terminal peptides at the glycosyl asparagine site and this route gave glycoproteins within 5–6 chemical steps from glycosyl asparagine thioacid. These methodologies successfully generated a series of glycoproteins with varying glycosylation positions and numbers. Using glycoproteins synthesized, we found that N-glycans enhance the binding affinity of glycoprotein with its receptor. This presentation introduces an interesting finding about how N-glycans behave in water.

(P203) Glycolipid antigen complexes for analysis of antigen presentation

Shunya Kikuchi[1], Nanako Kawate[1], Kazunari Ueki[1], Juri Takayama[1], Takanori Matsumaru[1], Yukari Fujimoto[1]

[1]*Graduate School of Science and Technology, Keio University,*

On antigen-presenting cell surfaces, both glycolipid- and peptide-antigens are presented by MHC or related molecules. As for the glycolipid antigen presentation, non-polymorphic MHC class I-like molecules such as CD1 are responsible. One of the CD1 family, CD1d, presents the glycolipid antigen to NKT cells via T cell receptors (TCR) of NKT cells to induce various cytokines, which include IFN-γ (Th1-type) and IL-4 (Th2-type). The representative glycolipid antigen, α -GalCer (KRN7000), strongly activates NKT cells, and we have found the lipid moiety strongly modulate the selective cytokine induction,[1)] and some of our lipid modified α -GalCer (KRN7000) were effective to modulate the immune balance including selective cytokine induction. In the present study, we firstly analyzed the selective immune modulation with various glycolipids, and designed complex molecules that combine peptide antigens with glycolipid antigens such as α -GalCer derivatives developed in our laboratory, for analysing the induction of acquired immunity. We then developed the synthetic methods for the designed antigen-complex molecules. For the preparation of the antigen-complex, we prepared both covalently or non-covalently complexed molecules. As for the covalently linked glycolipid-peptide complex, we firstly examined the linkers based on the biological activities. The linker was then introduced at 4 or 6-position of the galactose of α -GalCer derivatives. The modified peptide antigens (eg. cancer- or virus-derived antigens) was then synthesized for the reactions to link the lipid and peptide antigens. Utilizing these appropriately synthesized glycolipid and peptide antigens, the complex-molecules were synthesized and analyzed. We also analyzed the cellular behavior of the peptide- and glycolipid antigens with utilizing their molecular probes for further understanding of the antigen presentation.

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(P204) The Human Glycome Atlas Project for cataloging the human glycoproteome

Kiyoko Aoki-Kinoshita[1]

[1]Glycan and Life Systems Integration Center (GaLSIC), Soka University,

The Human Glycome Atlas (HGA) Project was launched in April, 2023, spearheaded by three institutes: the Institute for Glyco-core Research (iGCORE), Nagoya University and Gifu University, Tokai National Higher Education and Research System; the Exploratory Research Center on Life and Living Systems (ExCELLS), the National Institutes of Natural Sciences; and the Glycan and Life Systems Integration Center (GaLSIC), Soka University. This was the first time that a field in the life sciences was adopted by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) as a large-scale academic frontiers promotion project. This project aims to construct a knowledgebase of human glycans and glycoproteins as a standard for the human glycome. A high-throughput pipeline for comprehensively analyzing 20,000 blood samples in its first five years is planned, at which time an access-restricted version of this knowledgebase, called TOHSA, will be released. By the end of the final tenth year, TOHSA will provide a central resource linking human glycan data with other omics data including disease-related information.

(P205) Impact of O-glycosylation on Alzheimer's disease and glioma

Shinobu Kitazume[1]

[1]Fukushima Medical Universit,

The structural variety and functional role of protein O-glycosylation is not as well understood as protein N-glycosylation. In this symposium, I would like to emphasize that O-glycosylation plays an essential role in disease progression using the example of two key glycoproteins that play an essential role in the disease formation process of Alzheimer's disease and glioma.

We recently discovered that expression of amyloid b precursor protein 770 (APP770) in vascular endothelial cells contributes to amyloid β (Aβ) deposition in brain blood vessels, a condition known as cerebral amyloid angiopathy (CAA)(1). We therefore focused on endothelial APP770, in addition to neuronal APP695. Two types of APP, highly O-GalNAc glycosylated APP770 and hypo-O-glycosylated APP770, can be distinguished biochemically in endothelial cells, but only the former is cleaved for Aβ production(2). Although protein glycosylation is generally believed to precede cell surface trafficking, which is true for neuronal APP695, we unexpectedly observed that hypo-O-glycosylated APP770 is externalized to endothelial cell surfaces and transported back to the Golgi apparatus, where it then acquires additional O-glycans(3). Knockdown of genes encoding enzymes initiating APP O-GalNAc glycosylation significantly reduced Aβ production.

Gliomas are the most prevalent tumors of the central nervous system (CNS). Despite advances in medical technology, there are still limitations on glioma diagnosis and treatment. Protein tyrosine phosphatase receptor type Z (PTPRZ) is a membrane protein modified with several types of glycosylation, such as chondroitin sulfate, keratan sulfate N-glycans, O-GalNAc glycans, and O-Man glycans. We demonstrated that PTPRZ in gliomas have abundant levels of human natural killer-1 (HNK-1) capped O-Man core M2 glycans. As we recently discovered that soluble PTPRZ is markedly upregulated in the cerebrospinal fluid of glioma patients(4), we are currently working to develop an sPTPRZ sandwich ELISA. Finally, we discovered that deficiency of O-Man core M2 branching enzyme N-acetylglucosaminyltransferase IX leads to significant reduction of PTPRZ proteins and glioma tumor growth in xenograft models(5).

These results suggest that understanding of specific O-glycosylation processes may be beneficial for developing therapeutic and diagnostic glioma drugs.

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(P206) Genome Wide CRISPR Screening to Bioengineer a Safer Heparin

Jack Moore[1,2], Haruki Takeuchi[1,2], Ryan Weiss[1,2]

[1]Complex Carbohydrate Research Center, University of Georgia, [2]Biochemistry and Molecular Biology Department, University of Georgia,

Heparin is one of the most widely used drugs in the world, with approximately 12 million people in the US alone being administered heparin annually. Heparin acts as a potent anticoagulant due to its ability to activate antithrombin III (AT), a serine protease naturally found in the blood responsible for regulating the coagulation response. This interaction is facilitated by heparin's highly negative overall charge as well as a critical pentasaccharide sequence present along its repeating disaccharide

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backbone. While an effective anticoagulant, a major drawback to the use of heparin arises from the life-threating side effect of heparin induced thrombocytopenia (HIT), which occurs from an off-target interaction between heparin and the chemokine platelet factor 4 (PF4). Interestingly, heparin is only produced in mast cells and is predominately sourced from pig mucosa in China. Although heparin is exclusively produced in mast cells, a structurally analogous molecule known as heparan sulfate (HS) is ubiquitously expressed on the surface of all metazoan cells. Heparin and HS share the same repeating disaccharide backbone; however, they differ significantly in the quantity and patterning of various sulfation modifications. Due to their differences in composition, most notably due to a lack of a rare 3-*O*-sulfate group, HS does not possess the same anticoagulant capabilities of heparin. Both heparin and HS share the same biosynthetic machinery, yet the regulation of these enzymes and the assembly of heparin versus HS remains poorly understood. In this study, we developed genome-wide CRISPR screening strategies to identify factors that can regulate the expression of HS to enhance its interaction with AT and reduce its interaction with PF4, as a method to bioengineer a safer, recombinant form of anticoagulant heparin. From the screens, we identified previously studied genes essential for HS/heparin formation. Additionally, we uncovered novel candidate genes whose function is unknown relative to HS/heparin assembly. Regulatory factors identified from the genetic screens will be used to bioengineer a cell line that can produce a safer form of the drug heparin and will help elucidate the regulatory mechanisms for this vital complex polysaccharide.

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(P207) Identification of a novel bacterial galactose-3S preferring sulfatase using functional metagenomic screening

Julia Dreifus[1], Lea Chuzel[1], Chris Taron[1]

[1]*New England Biolabs, Ipswich, MA 01938, USA,*

Glycans are involved in many biological processes and play a key role in numerous diseases. The biological functions of glycans are dictated both by the arrangement of saccharide residues and the presence or absence of specific chemical saccharide modifications (termed post-glycosylation modifications, or PGMs). PGMs encompass a range of diverse chemical groups, including sulfate, phosphate, acetyl, methyl, and zwitterions and the distinct chemistry of each alters how glycans interact with surrounding molecules[1]. Consequently, changes in modification patterns can have severe impacts on health. For instance, mutations in some sulfotransferases cause congenital disorders, including muscular-skeletal abnormalities, deafness, and heart defects[1]. PGMs are technically challenging to identify and characterize, leaving them under-explored despite their importance. To this end, we are using a high-throughput functional metagenomics screening approach to discover novel bacterial enzyme specificities to facilitate the study of post glycosylation modifications.

In this study, we screened a human gut microbiome DNA fosmid library for an enzyme that can remove sulfate from the third carbon of galactose, a common modification found on N- and O-linked glycans in mammals and the influenza virus[2,3,4]. We identified a highly specific sulfatase that removes sulfate exclusively from galactose-3-sulfate. While this sulfatase is classified by bioinformatics as part of the S1_46 sulfatase subfamily, the only other biochemically defined family member specifically removes sulfate from the third carbon of *N*-acetylglucosamine[5,6]. Thus, this enzyme defines a new specificity attributed to this subfamily. This galactose-3-sulfatase described in this work contributes a novel specificity to a powerful enzymatic toolbox that will be used to facilitate the study of post-glycosylation modifications.

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(P208) Enhancing N-Glycopeptide Enrichment with Phosphorylcholine ZIC-HILIC Resins as a Complement to iSPE®-HILIC

Xu Yang[1], Asif Shajahan[1], Bhoj Kumar[1], Christian Heiss[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA,*

Glycoproteomic analysis on complex biological samples has become an essential tool in combating significant health threats. Mass spectrometry-based glycoproteomics has emerged as the primary method for this purpose, largely due to the growing

availability of glycopeptide enrichment techniques. One such technique, iSPE®-HILIC, a zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) resin, has been widely used for glycoproteomic studies, particularly with human serum samples. However, the potential biases of iSPE®-HILIC for broader sample types, such as the HEK293 cell line, have received little attention. Another ZIC-HILIC resin, featuring an exposed phosphorylcholine group, has been extensively used for C-reactive protein (CRP) enrichment. In this study, we have introduced a novel enrichment approach, termed phosphorylcholine enrichment (PCE), that employs this resin to complement iSPE enrichment. We evaluated the enrichment outcomes of various combinations of these two resins, using the already powerful Orbitrap Eclipse mass spectrometer. By applying PCE and iSPE on HEK293 cell samples complementarily, we achieved unbiased glycoproteome coverage. Notably, the iSPE-first approach, scavenged by PCE enrichment, increased unique N-glycopeptide detection in unfractionated HEK293 cell digest by a factor of 5.40 compared to unenriched sample. Our study highlights the importance of selecting an appropriate glycopeptide enrichment strategy tailored to the specific research objectives. This work has been supported by the US National Institutes of Health (R24GM137782 to PA); GlycoMIP, a National Science Foundation Materials Innovation Platform funded through Cooperative Agreement (DMR-1933525).

(P209) The Role of Flanking Charged Residues and Glycopeptide Substrate Conformation on the Activity of the Mucin Core Elongating Transferases

Collin Ballard[1], Haley Aharoni[1], Matthew Smutny[1], Hana Lee[1], Thomas Gerken[1]

[1]*Case Western Reserve University, School of Medicine, Department of Biochemistry, Cleveland, OH,*

Mucin-type O-glycosylation, henceforth O-glycosylation, is a complex and abundant post translational modification of secreted and membrane proteins. Mutations and differential expression of the enzymes involved in O-glycosylation are associated with many disease states including cancers. O-glycosylation is initiated by the GalNAc-T family of enzymes, which transfer an α-GalNAc from UDP-GalNAc onto the hydroxyls of serine and threonine. The Thr/Ser-O-GalNAc glycan is further elongated by the core elongating glycosyltransferases producing an array of complex structures. How the core transferases determine which O-GalNAcs are modified remains unknown, although flanking peptide charge may play a role, similar to what was observed for the GalNAc-Ts. To test this, we obtained a series of differently charged synthetic Thr-O-GalNAc glycopeptide substrates for use against the core transferases: C1GalT1 (which adds Galβ1,3-), B3GNT6 (which adds GlcNAcβ1,3-), ST6GalNAc-I, and -II (which adds NeuNAcα2,6-). We found that these transferases were indeed differentially sensitive to the charge of the synthetic glycopeptide, however, these results did not match their electrostatics. To confirm our results, studies were performed on chemoenzymatically produced Thr-O-GalNAc glycopeptides, generated by GalNAc-T1 acting on unglycosylated peptide substrates. These supposedly identical substrates gave significantly different results against the C1GalT1 and ST6GalNAc-I transferase compared to the synthetic glycopeptides. Interestingly, upon prior heating of the synthetic glycopeptides, their charge preferences changed to match those obtained from the chemoenzymatic approach. MS and NMR evidence suggests the synthetic glycopeptides may have an altered Thr-O-GalNAc and/or altered peptide conformation, different from the more native like chemoenzymatic glycopeptides, which can be annealed to a more "native" like conformation upon heating. To support this, we found the change in activity upon heating the synthetic glycopeptide can persist for days to tens of months, before reverting back to the non-heated activities, depending on the sequence of the synthetic glycopeptide. The crystal structure of C1GalT1 bound to a glycopeptide substrate supports the idea that glycopeptide conformation can play a role in its activity showing a unique strained conformation of the bound Thr-O-GalNAc. Interestingly, ST6GalNAc-II and B3GNT6 do not show such differences between the synthetic and chemoenzymatic derived substrates suggesting that these transferases are not sensitive to glycopeptide conformation. Overall, we have shown that flanking substrate charge variably affects the activities of the core glycosyltransferases and that a subset of these enzymes are uniquely sensitive glycopeptide substrate conformation. These studies reveal a previously unknown conformational sensitivity that is unique to the core transferases which will lead to a better understanding of their specificities.

Supported by NIH grant R01 GM113534.

Acknowledgements: We thank Kelley Moremen at the Univ. of Georgia, and Ramon Hurtado-Guerrero at the Univ. of Zaragoza for supplying the core transferases and Lance Wells and Robert Bridger at the Univ. of Georgia for MS analysis.

(P210) Development and Comparison of Methods for Identification and Quantification of Glycosphingolipids (GSLs)

Mehrnoush Taherzadeh Ghahfarrokhi[1], Stephanie Archer-Hartmann[1], Christian Heiss[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center (CCRC), University of Georgia, Athens, GA, USA,*

Glycosphingolipids (GSLs) are a group of glycolipids with a glycan head glycosidically linked to the C-1 hydroxyl group of a ceramide tail. While ceramides exhibit variations in chain length, hydroxylation, and saturation, the primary structural and

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functional categorizations are based upon the diverse glycans. These glycans differ in type, number, composition, and linkage positions of their monosaccharides. GSLs play pivotal roles in various biological processes, including cell adhesion, migration, signaling, proliferation, endocytosis, intracellular transport, inflammation, and apoptosis. These functional roles are dependent on their structural features. Thus, precise identification and quantification of GSLs hold significant importance. This study aims to adopt a comparative approach for identification and quantification of GSLs. To this end, two methods for analyzing GSLs, each starting with 106 HEK cells, were compared. In the first experiment, isolated GSLs were permethylated for higher sensitivity and analyzed using nESI-MS/MS. In the second experiment, GSLs were first cleaved with endoglycoceramidase (EGCase) II, and the released glycans were labeled with procainamide and analyzed with UPLC-FLR-MS/MS. Notably, chromatography in the second method facilitated the separation of isomeric glycoforms that were indistinguishable through the other method. Also, this method showed higher sensitivity, identifying 2 new glycoforms. The boost of sensitivity in analysis of GSLs in the second method is a result of removing the ceramide, thus reducing the heterogeneity associated with its ceramide variety. The EGCase II released glycans comprised both neutral and sialylated species with varying sizes. Optimization of the reaction and cleanup improved the coverage and sensitivity of this method. MS/MS fragmentation was used for structure assignment confirmation in both methods. Since permethylation and procainamide labeling changes the m/z value of GSLs and glycoforms, the limited existing online databases and tools for lipids and metabolites are of small use for data interpretation. A database of possible m/z values for GSLs and glycoforms was compiled for assistance in data interpretation. Finally, we found that utilization of an internal standard (GM3-d9) can be helpful for quantification of permethylated GSLs.

(P211) Developing a cell-free platform for engineering bacterial oligosaccharyltransferases

Derek Wong[1,2,3], Katherine Warfel[1,2,3], Zachary Shaver[1,2,3], Sarah Sobol[1,2,3], Regina Fernandez[1,2,3], Michael Jewett[4]

[1]*Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL,* [2]*Chemistry of Life Processes Institute, Northwestern University, Evanston, IL,* [3]*Center for Synthetic Biology, Northwestern University, Evanston, IL,* [4]*Department of Bioengineering, Stanford University, Stanford, CA,*

Glycoconjugate vaccines, composed of a pathogen specific polysaccharide antigen (such as an O-antigen or capsular polysaccharide) linked to an immunogenic carrier protein, are a promising strategy to protect against bacterial infections. Current manufacturing processes for conjugate vaccines involve non-specifically chemically conjugating the purified polysaccharide of interest to a recombinantly expressed and purified carrier protein. While functional, these processes are expensive, require growing the pathogenic bacterial species, and result in a heterogenous product. Recent work in the field have developed alternative *in vivo* and cell-free methods for producing conjugate vaccines by using oligosaccharyltransferases (OSTs), such as PglB from *C. jejuni,* to site-specifically transfer polysaccharide antigens to carrier proteins of interest. By using glycoengineered *E. coli* strains or glycan enriched cell-free lysates, these simpler methods circumvent many of the challenges associated with current chemical conjugation manufacturing processes. Limiting further adoption of these methods, however, is the limited glycan substrate scope of known OSTs for polysaccharides of high interest bacterial pathogens. In this work, we create a cell-free, *in vitro* platform for expressing and testing OST variants for their ability to transfer glycans of interest. To begin, we demonstrate that OSTs expressed in nanodisc supplemented cell-free protein synthesis (CFPS) reactions are capable of transferring O-antigens and capsular polysaccharides. Next, we couple our CFPS based workflow with AlphaLISA, an in-solution bead-based ELISA assay, for detection of successful transfer of glycans onto a target protein. Finally, we demonstrate the utility of our workflow for rationally screening libraries of OSTs for transfer of a desired glycan antigen. In total, we expect that our workflow will enable the characterization of OSTs of interest as well as accelerate the timeline for development of novel conjugate vaccines.

(P212) Squaryl group-modified UDP Analogs as novel inhibitors of UDP-Glc:glycoprotein glucosyltransferase (UGGT)

Junpei Abe[1], Yusaku Sasae[1], Yoichi Takeda[2], Takashi Kikuma[2], Yasuhiro Kajihara[1,3], Yukishige Ito[1,3]

[1]*Graduate School of Sciences, Osaka University,* [2]*College of Life Science, Ritsumeikan University,* [3]*FRC, Graduate School of Science, Osaka University,*

A majority of newly synthesized proteins are glycosylated in eukaryotic cells and the biological activity of these glycoproteins can be exhibited due to their correct folding. Especially in the case of N-linked glycoprotein, a specific folding system exists in the endoplasmic reticulum (ER) called as an ER-quality control system (ERQC). In ERQC, the Calnexin/Calreticulin (CNX/CRT) system has a central role in N-linked glycoproteins folding by recognizing Glc of G1M9 (Glc-Man_9-$GlcNAc_2$) in glycoproteins. However, N-linked glycoproteins sometimes have not been folded completely. UDP-Glc:glycoprotein glucosyltransferase (UGGT) works as a sensor to detect the unfolded N-linked glycoproteins, which makes the unfolded glycoproteins enter the CRT/CNX system again by glucosylation at the outermost A-branch mannose of M9-glycoprotein. UGGT is related to some biological events, but that's extent to the ERQC has been not investigated well. A potent and selective UGGT inhibitor is desirable to

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elucidate that. There are some molecules with UGGT inhibitory activity, such as unfolded protein with or without N-linked glycan and N-linked glycopeptide. Regarding small molecules with UGGT inhibitory activity, a few examples are known. Glycosyltransferase uses sugar nucleotide as a substrate and the resultant nucleotide usually binds to the glycosyltransferase known as product inhibition. The same situation can be observed in UGGT. As nucleotide moiety has an inhibitory activity, the strategy to create glycosyltransferase inhibitors is often to replace or modify a sugar or diphosphate part of a sugar nucleotide. In the case of UGGT, structure information derived from co-crystallization with UDP and the catalytic domain of *T. Thermophilus* UGGT of which similarity is high among some species can be accessible, leading to modification of nucleoside moiety rationally. As sufficiently potent UGGT inhibitor is scarce, we explored the possibility of creating UGGT inhibitor. To date, we elucidated squaryl group-modified UDP analog possessing 2’-amino group and 2-ethanolamino group in squaramide exhibited higher UGGT inhibitory activity than UDP. This time further structure activity relationship study (SAR) especially in uracil moiety was carried out. The result of modifications of uracil moiety will be discussed.

(P213) Chemo-enzymatic N-glycan remodeling for homogeneous asymmetric glycosylated IgG and influence of N-glycosylation on FcγRIIIa binding affinity

Methanee Hiranyakorn[1], Saeko Yanaka[2,3], Atsuji Kodama[3], Shogo Iwamoto[4], Asako Hoshinoo[4], Koichi Kato[2,3], Shino Manabe[1,5]

[1]School of Pharmacy and Pharmaceutical Sciences, Hoshi University, [2]Institute for Molecular Science (IMS), [3]Exploratory Research Center on Life and Living Systems (ExCELLS), [4]Fushimi Pharmaceutical Co. Ltd., [5]Graduate School of Pharmaceutical Sciences & Faculty of Pharmaceutical Sciences, Tohoku University,

Immunoglobulin G (IgG) is an essential antibody used in therapeutics, consisting of a Fab region for antigen recognition and an Fc region that interacts with effector molecules. The Fc region comprises two identical chains, each containing conserved *N*-glycosylation. *N*-linked glycosylation plays a crucial role in the structural and functional properties of IgGs. However, the complexity of the biosynthetic pathway results in highly variable *N*-glycan structures in antibodies, both symmetric and asymmetric. Nonetheless, the relationship between glycan structure and antibody functions remains unclear due to the heterogeneous nature. To elucidate the structural relationship between *N*-glycan and antibody activities, homogeneous *N*-glycan carrying IgG is highly desired. Glycan remodeling strategies using glycan oxazoline and endo-b-*N*-acetylglucosaminidases mutant have been employed to overcome this difficulty. Nevertheless, previous reports had the limitation of preparing only symmetric glycan type antibodies. (H. Wei *et al. J. Am. Chem. Soc.*, 134, 12308 (2012); C.W. Lin *et al. PNAS*, 112, 10611 (2015)) Therefore, the potential of IgG with asymmetric *N*-glycan remains unclear due to the lack of an established methodology. Here we provided a new methodology for producing asymmetric glycosylated IgG. By combining the advanced purification method and chemo-enzymatic reaction, we successfully prepared homogeneous glycosylated rituximab. Our approach enables the preparation of both asymmetric and symmetric glycan carrying rituximab. Additionally, we investigated the relationship of the *N*-glycan structure and binding affinity of rituximab to FcγRIIIa using FcγRIIIa column chromatography and Surface Plasmon Resonance (SPR). Our data revealed that the length of the glycan on rituximab influenced its retention time on the FcγRIIIa column, consistent with SPR data, indicating a relationship between glycan length and binding affinity to FcγRIIIa. Furthermore, the presence and positioning of sialic acid also affected the binding affinity. Our results would provide insights into the underlying structural basis of biological processes and opening new avenues for utilizing glycans to enhance therapeutic efficacy.

(P214) Preclinical development of heparan sulfate oligosaccharides for drug induced liver injury

Katelyn Arnold[1,2], Yongmei Xu[1], Chaoying Yin[1], Zhangjie Wang[2], Jian Liu[1]

[1]Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA, [2]Glycan Therapeutics Corp, Raleigh, NC, USA,

Many drugs including acetaminophen (APAP), antibiotics, and anti-tuberculosis drugs have the potential to cause drug induced liver injury. Extracellular high mobility group box 1 (HMGB1) is central to the damaging sterile inflammation response that contributes to drug induced liver injury. There are no therapeutics available targeting HMGB1, and few options are available for drug induced liver injury. We recently reported that synthetic heparan sulfate (HS) inhibits HMGB1-mediated inflammation in mouse models of peritonitis and APAP induced acute liver failure (*Sci Transl Med*. 2020;12(535)). Importantly, we showed that synthetic HS was protective when administered 3 hours post-APAP overdose in contrast to the standard of care, N-acetyl cysteine. We are pursuing synthetic HS for APAP induced liver injury indication in collaboration with Glycan Therapeutics Corp. Our goal is to identify a smaller oligosaccharide for preclinical development.

In this work, we demonstrate the preclinical development activities of synthetic HS including structure, dose, and injection schedule optimization as well as pharmacokinetic studies in mice. The previously reported 18-mer required a lengthy synthesis

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route due to its size. We sought to find a shorter compound to reduce the synthesis burden and increase the scale rapidly. We selected smaller analog compounds that had a similar structural pattern. We first tested for HMGB1 immunoprecipitation to confirm binding. HMGB1 binding compounds were screened in a mouse model of APAP overdose to determine effectiveness. Two compounds, 14- and 16-mer-6S, decreased plasma ALT in a dose-dependent manner, with 16-mer-6S achieving a significant decrease when given at a high dose. Next, we tested a smaller compound, GLY-202, that has a higher sulfation density and binds to HMGB1. GLY-202 significantly decreased plasma ALT when given 30 min after APAP overdose. Based on pharmacokinetic studies of GLY-202 and its similarities to 18-mer, we elevated the dose and gave a single injection 3 hours post-APAP. Interestingly, this strategy resulted in a greater fold reduction of plasma ALT compared to dosing 18-mer shortly after APAP overdose and again 12 hours later. This result suggests that plasma HMGB1 has a large role in damaging inflammation early on in liver injury. Inhibiting HMGB1 at this stage is sufficient for liver protection. Future efforts include performing IND-enabling studies. HMGB1 has been implicated in many acute and chronic liver diseases, therefore an HMGB1 inhibitor may have broad utility in hepatology.

(P215) A Lectin-Drug Conjugate CRISPR Screen Identifies Sortilin as the Lysosomal Trafficking Receptor for Galectin-1

Justin Donnelly[1], Roarke Kamber[2], Simon Wisnovsky[3], David Roberts[1], Egan Peltan[1], Michael Cory Bassik[1], [1]

[1]Stanford University, [2]University of California, San Francisco, [3]University of British Columbia,

Galectins are a family of mammalian glycan-binding proteins that have been shown to regulate an array of critical cellular functions, such as migration, apoptosis, and receptor-ligand binding. Despite their clear importance, the molecular mechanisms of galectins' activity – the specific binding partner(s) and cellular context necessary for a particular biological effect – remain understudied. Along with the biochemical challenges inherent to the study of lectin-glycan interactions, investigations into the mechanisms of galectin function are complicated by the fact that several members of this family, such as galectin-1, exhibit both glycan-dependent and glycan-independent functions. To make matters worse, galectin-1 can be found in the endomembrane system, nucleus, or cytosol, as well as on the cell surface. Little is known of how galectin-1's trafficking between different compartments, except that it is secreted and internalized via unconventional and poorly-understood mechanisms. Here, we develop a novel lectin-drug conjugate based on galectin-1 and utilize this construct in a genome-wide CRISPR screen that investigates galectin-1's intracellular trafficking. We demonstrate that a glycan-dependent interaction with the receptor sortilin plays a key role in determining the fate of internalized galectin-1, showing that sortilin strictly regulates galectin-1 trafficking to the lysosome. We also highlight the potential utility of this interaction to deliver other molecules such as exogenous proteins and siRNAs to this key organelle.

(P216) Design of a Novel Chimeric Heparin-like Anticoagulant that resists Heparanase Biodegradation

Eduardo Stancanelli[1], Yongmei Xu[1], Yi-En Liao[1], Elliot L. Chaikof[2], Jian Liu[1]

[1]Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Rm 1044, Genetic Medicine Building, Chapel Hill, North Carolina 27599, United States, [2]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 3 Blackfan Circle, CLS-11090, Boston, Massachusetts 02215, United States,

Heparan sulfate and heparin are highly sulfated polysaccharides. Heparin is commonly used as anticoagulant because its capacity of tightly binds and inhibits the activities of factors Xa and IIa (also known as thrombin) to prevent blood clot formation. Heparanase, an endoglycosidase mainly found in platelets, mast cells and leukocytes, which is capable of degrading heparan sulfate chains and hence heparin and LMWH, may contribute to heparin resistance, a common occurrence in cancer patients, by being overexpressed by tumor cells.

Here, we report the synthesis of a novel, size-defined oligosaccharide assembled by click chemistry reaction that displays both anti Xa and anti IIa activity and shows resistance towards Heparanase. A chemoenzymatic approach, involving glycosyltransferases, sulfotransferases, and C_5-epimerase, was used to prepare clickable building blocks with the domain that specifically binds AT (Antithrombin). Before oligosaccharide building blocks were assembled, we introduced a hybrid CS (chondroitin sulfate) domain to prevent Heparanase degradation. Anti-Xa and anti-IIa activity measurements revealed that both anti-IIa and anti-Xa activities are well preserved after Heparanase digestion. HPLC and ESI-MS analysis verified the hypothesis about Heparanase resistance showing the intact oligosaccharide.

The results from this study demonstrate the ability to synthesize customized HS oligosaccharides to probe the structure and activity relationships and enhance their properties by generating unnatural HS-CS chimeric building blocks, offering a novel class of glycosaminoglycan molecules with potential clinical efficacy.

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(P217) Serum glycopeptide signatures associated with reduced clinical benefit of immune checkpoint inhibitor therapy are induced by cytokines that modulate expression of glycosylation-related genes in the liver

Frederico da Silva[1], Chih-Wei Chu[1], Chad Pickering[1], Gregg Czerwieniec[1], Gege Xu[1], Tomislav Čaval[1], Daniel Serie[1], Flavio Schwarz[1]

[1]*InterVenn Biosciences, 2 Tower Pl 5th Floor, South San Francisco, CA 94080,*

The remarkable success of immune-checkpoint inhibition (ICI) in metastatic melanoma treatment has underscored the value of strategies that enhance the immune system's response against cancer. However, as the prolonged benefits are observed in only approximately half of the patients and are often accompanied by significant adverse events, there is a need for predictive biomarkers and patient stratification strategies. While increased PD-L1 expression and tumor mutational burden have shown some correlation with response to ICI treatment in certain cancers, these markers have limited predictive capability in metastatic melanoma.

By employing a glycoproteomics platform that combines targeted mass spectrometry with data analysis powered by artificial intelligence on pre-treatment plasma samples collected from 202 metastatic melanoma patients who were administered anti-PD-1 monotherapy or anti-PD-1/anti-CTLA-4 combination therapy, we previously identified biomarkers that distinguish individuals likely to experience extended survival from those less likely to benefit from therapy and discovered a fucosylation signature in N-linked glycoproteins of patients with reduced overall survival. In-depth analysis of the fucosylated markers using bottom-up glycoproteomics revealed that the majority were core fucosylated, while a few presented with an outer-arm fucose modification. As the majority of the identified fucosylated serum markers are liver-derived glycoproteins, we hypothesized that inflammatory mediators, possibly produced by cells in the tumor microenvironment and released in the circulation, may act as drivers of fucosylation changes in the liver. We measured cytokines in the serum of the melanoma patients and observed that a set of molecules including TGF-β1, IL-1β, IL-6, IFN-β and TNFβ were differentially expressed in patients classified based on a glycopeptide-based model. Treatment of human hepatocytes *in vitro* with these cytokines increased cell surface fucosylation, which could be prevented by antibodies that blocked the interaction of the cytokines to their receptors. Interestingly, gene expression analysis revealed that while TNF-α upregulated the expression of the GDP-Fucose transporter *Slc35c1*, TGF-β1 induced *FUT8* expression and IL-1β upregulated both *Slc35c1* and *FUT3 in vitro*.

Taken together, these results indicate that elevated levels of inflammatory markers in the serum of melanoma patients not benefiting from ICI therapy can function as molecular drivers for the synthesis of fucosylated proteins by inducing the expression of fucosylation-related genes in hepatocytes. We propose that upregulation of fucosylation is a result of a hepatic reprogramming mechanism induced by cancer.

(P218) Increased 3-*O*-sulfated heparan sulfate in Alzheimer's disease brain is associated with genetic risk gene HS3ST1

Zhangjie Wang[1], Vaishali N. Patel[2], Xuehong Song[3], Yongmei Xu[1], Andrea M. Kaminski[4], Vivien Uyen Doan[1], Guowei Su[5], Vijayakanth Pagadala[5], Chunyu Wang[6], Lars C. Pedersen[4], Lianchun Wang[3], Matthew P. Hoffman[2], Marla Gearing[7], Jian Liu[1]

[1]*Eshelman School of Pharmacy, University of North Carolina,* [2]*Matrix and Morphogenesis Section, National Institute of Dental and Craniofacial Research,* [3]*Department of Molecular Pharmacology & Physiology, Bryd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida,* [4]*Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health,* [5]*Glycan Therapeutics Corp.,* [6]*Department of Biological Sciences, Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute,* [7]*Department of Pathology and Laboratory Medicine and Department of Neurology, Emory University School of Medicine*

HS3ST1 is a genetic risk gene associated with Alzheimer's disease (AD) and overexpressed in patients, but how it contributes to the disease progression is unknown. we report the analysis of brain heparan sulfate from AD and other tauopathies using a new LC-MS/MS method. A specific 3-O-sulfated heparan sulfate (HS) displayed 7-fold increase in the AD group ($n = 14$, $P < 0.0005$). Analysis of the HS modified by recombinant sulfotransferases and HS from genetic knockout mice revealed that the specific 3-O-sulfated HS is made by 3-O-sulfotransferase isoform 1 (3-OST-1), which is encoded by the HS3ST1 gene. A synthetic tetradecasaccharide (14-mer) carrying the specific 3-O-sulfated domain displayed stronger inhibition for tau internalization than a 14-mer without the domain, suggesting that the 3-O-sulfated HS is used in tau cellular uptake. Our findings suggest that the overexpression of HS3ST1 gene may enhance the spread of tau pathology, uncovering a new therapeutic target for AD.

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(P219) Synthesis of heparin oligosaccharides with both anti-factor Xa and anti-factor IIa activities

Yongmei Xu, Eduardo Stancanelli, Guowei Su[1], Jian Liu

[1]*Glycan Therapeutics, 617 Hutton Street, Raleigh, North Carolina, USA,*

Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina, USA

Heparin is used clinically to treat clotting disorders by inhibiting the activities of factors Xa and IIa. As an animal-sourced product, heparin's supply chain reliability is a concern for regulatory agencies. Heparin has strong anticoagulant activity, fast clearance rate, and is convenient for doctor monitoring the anticoagulant activity. Here, we synthesized authentic oligosaccharides of 16–22 residues using chemoenzymatic method, and mimic oligosaccharides of 18–24 residues clicking both domain structures. Factor IIA assay reveals an authentic octadecasaccharide (18-mer) with GlcNac-GlcA-GlcNac-GlcA linkage between antithrombin and thrombin domains displays anti-factor IIA activity; the thrombin domain longer than 8 residues with N-sulfate and either 2-O-sulfate or 6-O-sulfate displays anti-factor II activity. In vitro and ex vivo experiments demonstrate that both authentic 18-mer and clicked 20-mer have good anticoagulant activity.

(P220) Novel enzymes converting group A red blood cells to the universal donor group O

Marco Moracci[1], Nicola Curci[1,2], Roberta Iacono[1], Dorotea Raventos Segura[4], Michele Cillo[5], Beatrice Cobucci-Ponzano[2], Andrea Strazzulli[1,3], Antonio Leonardi[5], Lars Giger[4]

[1]*Department of Biology, University of Naples "Federico II", Complesso Universitario di Monte S. Angelo, Via Cinthia 21, Naples, 80126, Italy,* [2]*Institute of Biosciences and BioResources, National Research Council of Italy, Via P. Castellino 111, Naples, 80131, Italy,* [3]*NBFC, National Biodiversity Future Center, Palermo 90133, Italy,* [4]*Novozymes A/S; Biologiens vej 2, 2800 Kgs. Lyngby Denmark,* [5]*Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", Via Sergio Pansini, 5, Naples 80131, Italy,*

Glycoside hydrolases (GHs) have been employed for industrial and biotechnological purposes and often play an important role in new applications. The red blood cell (RBC) antigen system depends on the composition of oligosaccharides on the surface of erythrocytes, thus defining the ABO blood type classification. Incorrect blood transfusions may lead to fatal consequences, making the availability of the correct blood group critical. In this regard, it has been demonstrated that some GHs could be helpful in the conversion of A and B blood types to produce group O universal donor blood. GHs belonging to the GH109 family are of particular interest for this application due to their ability to convert blood from group A to group O [Liu et al. 2007; Rahfeld and Withers 2020]. We report on the biochemical characterization of three novel GH109 enzymes (NAg68, NAg69 and NAg71) and the exploration of their ability to produce enzymatically converted RBCs (ECO-RBC). The three enzymes showed superior specificity on pNP-α-*N*-acetylgalactosamine compared to previously reported GH109 enzymes. These novel enzymes were able to act on purified antigen-A trisaccharides and produce ECO-RBC from human donor blood. NAg71 converted type A RBC to group O with increased efficiency in the presence of dextran compared to a commercially available GH109, previously used for this application. Our data demonstrates that novel CAZymes are powerful tools for design and improvement of biomedical applications and demonstrates the importance of detailed characterization of the enzymes to exploit their potential as biocatalysts. Members of the GH109 family represent remarkable and underexplored candidates for enhancing ECO-RBC production technology [Curci et al. 2023].

Curci N, Iacono R, Raventos Segura D, Cillo M, Cobucci-Ponzano B, Strazzulli A, Leonardi A, Giger L, Moracci M. Novel GH109 enzymes for bioconversion of group A red blood cells to the universal donor group O. New Biotechnology 2023 *in press* https://doi.org/10.1016/j.nbt.2023.08.002.

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(P221) IBISBA: the european infrastructure of industrial biotechnology and synthetic biology for carbohydrate active enzymes discovery and application

Marco Moracci[1], Mauro Di Fenza[2], Beatrice Cobucci Ponzano[2], Giuseppe Perugino[1], Ezio Ricca[1]

[1]*Dept. of Biology, University of Naples 'Federico II', Naples, Italy,* [2]*Institute of Biosciences and BioResources-National Research Council of Italy, Naples, Italy*

The Industrial Biotechnology Innovation & Synthetic Biology Accelerator (IBISBA- www.ibisba.eu) is a pan-European research infrastructure dedicated to industrial biotechnology. We provide a single access point to researchers from academia and industry

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across the globe to integrated services for end-to-end bioprocess development. We federate 21 European universities and research & technology organisations to provide researchers from academia and industry across the globe with integrated services for all stages of bioprocess development projects.

IBISBA vision is to make industrial biotechnology the driver of the global circular bioeconomy transition. Using microorganisms and enzymes to convert biomass and waste into products and services, biotechnology drives low impact manufacturing processes in many sectors including energy, agriculture, forestry, and pharma. In other words, industrial biotechnology is set to become to the circular bioeconomy what industrial chemistry has been to the petro-economy.

IBISBA adopts an integrated approach to bioprocess development, connecting disciplinary expertise and targeting requisite technical specifications for bioprocessing right from the start. IBISBA acts as a superhub, bringing capabilities and platforms together. Like an airline alliance, IBISBA provides its users with a more seamless R&D experience and aims to support every step of user projects. Similarly, all of IBISBA's research infrastructures components agree to cooperate in order to take users further, improving interoperability and sharing standards and resources. Accordingly, in the IBISBA approach, biocatalysts and bioprocesses are designed in concert, ensuring that the biocatalyst reaches the right bioprocess performance under actual process conditions, and that the process environment is fully adapted to enhance biocatalyst performance and deliver quality products. IBISBA partners offer five categories of services: protein discovery and engineering, production strain development, bioprocess development & optimisation, and omics & analytics. In these regards, IBISBA Italy contributes with their expertise and facilities to the discovery and engineering of carbohydrate active enzyme for a variety of applications in the field sustainable agriculture and food production, pharma and biomedicine.

Reference

[1] IBISBA Annual Report 2021. DOI: 10.34701/ibisba.1.document.34.1.

(P223) Improvements in glycoproteomics through architecture changes to the Tribrid MS platform

Nicholas M. Riley[1], Jingjing Huang[2], David Bergen[2], Amanda E. Lee[2], Rafael D. Melani[2], William D. Barshop[2], John E.P. Syka[2], Jesse D. Canterbury[2], Vlad Zabrouskov[2], Graeme C. McAlister[2], Christopher Mullen[2]

[1]*Department of Chemistry, University of Washington, Seattle, Washington, USA,* [2]*Thermo Fisher Scientific, San Jose, California, USA*

Recent hardware changes introduced on the Orbitrap Ascend Tribrid MS include dual ion routing multipoles (IRMs) that can be used to parallelize accumulation, dissociation, and mass analysis of three separate ion populations. Here we explore how this architecture improves N- and O-glycopeptide characterization by increasing scan acquisition speeds without sacrificing spectral quality. The balance between scan speed and MS/MS product ion signal-to-noise is especially important in glycoproteomics. Complexities of glycopeptide fragmentation necessitate large precursor ion populations, and consequently, long ion accumulation times, for quality MS/MS spectra. To compound matters further, dissociation methods like electron transfer dissociation (ETD) that benefit glycopeptide characterization come with overhead times that also slow down scan acquisition. Conversely, heterogeneity inherent to glycosylation means that any given retention time during an LC-MS/MS analysis may contain numerous glycopeptide species to target through data-dependent acquisition. Often duty cycle is sacrificed to some degree, which results in higher quality spectra of abundant species but leaves other precursor ions under-sampled. We analyze mixtures of N- and O-glycopeptides to show that 20–30% more MS/MS scans can be acquired when parallelizing three ion populations using the dual IRMs of the Orbitrap Ascend. This translates to 10–20% gains in glycopeptide identifications depending on dissociation type(s), scan acquisition schemes, and method parameters (e.g., precursor ion accumulation and reaction times). Focusing on O-glycopeptide analysis with ETD-based methods, we also explore how acquisition rates and ion-ion reaction times affect identifications and product ions generation. We show what parameters need to be considered in O-glycopeptide characterization to generate c- and z-type ions that can be used for O-glycosite localization while also maximizing scan acquisition rates to improve total site-localized O-glycopeptide identification. In all, we show how architectural changes to the Tribrid MS platform benefit glycoproteomic experiments by parallelizing scan functions to minimize overhead time and improve sensitivity.

(P224) Large-scale and Site-specific Mapping of the Murine Brain O-Glycoproteome with IMPa

Suttipong Suttapitugsakul[1], Yasuyuki Matsumoto[1], Rajindra P. Aryal[1], Richard D. Cummings[1]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA,*

O-glycosylation plays important roles in cells by affecting protein functions, properties, interactions, and activities. The study of the O-glycoproteome for the brain or other organs has been complicated due to many factors, such as; the lower relative abundance of O-glycosylation in mammalian cells compared to that of N-glycosylation, the difficulties in identifying sites of O-glycosylation, as O-glycans are often clustered together within a domain; the lack of a consensus site for O-glycosylation such as occurs for N-glycosylation; and unlike PNGaseF used in N-glycoproteomics analysis, there is no common enzyme to deglycosylate O-glycans and generate a common tag for site determination. Technologies including SimpleCell or EXoO have

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been reported, but they do not address the native O-glycoprotein state in cells. Immunomodulating metalloprotease (IMPa) from *Pseudomonas aeruginosa* has been identified recently and shown to efficiently cleave O-glycoproteins and glycopeptides N-terminal to a serine or threonine residue containing an O-glycan, including core 1 O-glycans, sialylated core 1 O-glycans, the Tn antigen, and other O-glycans with varying complexity, with minimal influence from amino acids adjacent to the glycosylation site. Here we use IMPa to globally and site-specifically identify O-glycoproteins and glycosylation sites from the murine brain and several cultured cell types. Glycopeptides are first conjugated to a solid support and IMPa is used to cleave O-glycopeptides for intact glycoproteomics analysis with LC-MS/MS. We developed two approaches, one for total O-glycoproteomics analysis and the other for Tn-focused experiments by employing different methods to pre-enrich glycopeptides. The first approach targets the total O-glycoproteome in cells and tissues through glycopeptide enrichment with RAX SPE columns, which has been shown to be beneficial for intact O-glycopeptide enrichment, whereas the second method employs VVA enrichment for Tn-glycopeptide separation. This further ensures that the glycopeptides are modified with GalNAc, and not the dynamic O-GlcNAc glycan, which is not recognized by VVA. We employed HEK293 cells with various O-glycans or SimpleCell HEK293 cells with the uniformly expressed Tn antigen to test the global glycoproteomics analysis. We documented the importance of IMPa in the localization of O-glycans as well as the use of EThcD for O-glycopeptide analysis. We were successful in using these approaches to identify hundreds of different glycoproteins in the O-glycoproteome of the mouse brain. We also compared the single-shot analysis with the extensive fractionation approach, which improved the coverage by ∼3 times. We discovered that mouse brain O-glycoproteins are mostly modified with sialylated core 1 O-glycoproteins, which would have been missed or interpreted as core 1 O-glycan or the Tn antigen by other previously reported methods. Furthermore, we discovered the presence of Tn-containing glycoproteins in mouse brain, albeit at relatively low abundance. We further applied these methods to quantify changes in the O-glycoproteome in human brain across the Alzheimer's disease spectrum. Our work investigates the O-glycoproteome in its most native state, including the Tn and sialylated glycoproteome.

(P225) Glycan Microarray Services and Bioinformatics through the National Center for Functional Glycomics

Jamie Heimburg-Molinaro[1], Akul Y. Mehta[1], Catherine A. Tilton[1], Sandra F. Cummings[1], Lauren Pepi[1], Richard D. Cummings[1]

[1]*Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School, Boston, MA,*

The National Center for Functional Glycomics (NCFG) at BIDMC at Harvard Medical School (HMS) is an R24 National and Regional Resource Center, funded by the National Institutes of Health, NIGMS. The NCFG is an outgrowth of the successful Consortium for Functional Glycomics (CFG) glycan microarray resources. The overall goals of the NCFG focus on providing needed resources and technologies for defining protein-glycan interactions to the community. Our central discovery platform is glycan and glycopeptide microarrays, with glycans derived from chemo-enzymatic synthesis (*defined microarrays*) and natural animal sources (*shotgun microarrays*), and microbially-derived components. A set of glycan components are in the process of being translated to the Luminex platform to allow for additional binding capabilities. Additional linker strategies are in development to allow such features as reversibility, bifunctionality, and natural amino acid linkages. We hope to soon offer a *label-free method* for binding assays. We maintain legacy array data in addition to newly generated data, and work with GlyGen to synchronize datasets and allow for integration of new datasets into the repository. We also develop and utilize bioinformatics capabilities such as: *GLycan Array Dashboard (GLAD)* which provides tools for analyzing and comparing glycan array data and visualization capabilities; *GlycoGlyph* which enables glycan drawing with SNFG formatting with ease, export capabilities, and links to other databases and features; *Glybrary*, a newly developed comprehensive database for inputting and linking sample and assay details, connecting datasets, and tracking projects, with the goal of replacing the stagnant *CFG* website. These programs are or will be publicly accessible through the new NCFG website- https://research.bidmc.org/ncfg/

The paramount service offered through the NCFG is the analysis of glycan binding proteins on our collection of glycan microarrays, as fee-for-service and through collaborative research. The number, diversity, and biological relevance of both defined and shotgun glycan microarrays are continuously growing. Requests for any defined or shotgun microarrays and other printing projects can be directed to the NCFG. Tangential work through the BIDMC Glycomics Core and Cummings Lab includes comprehensive Glycomic analyses using advances mass spectrometry methods, and development of robust anti-glycan reagents using the lamprey system. Overall, we focus on technologies aimed at defining cellular glycomes important in human biology and disease, and we aim to support the community in their endeavors into defining protein-glycan interactions. *R24GM137763*

(P226) Next generation chemical tools for multi-modal glycome analysis

Ulla I.M. Gerling-Driessen[1], Marc D. Driessen[2]

[1]*Department of Macromolecular Chemistry, Heinrich-Heine-University, Duesseldorf, Germany,* [2]*Institute for Molecular Medicine, Proteome Research, Heinrich-Heine-University, Duesseldorf, Germany*

Analysis of the various components of the glycome can be very challenging due dynamic changes in glycan heterogeneity and composition. To alleviate this problem, enrichment of target glycoconjugates, based on covalent attachment procedures is one

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commonly applied strategy. Bioorthogonal chemistry is one of the most used approaches for the covalent attachment of analytical probes to chemically modified glycans. Many of the commonly employed bioorthogonal probes however are designed for use in a specific type of experiment (e.g., either imaging or crosslinking or enrichment etc.). This can make multiplex or multi-use applications difficult and expensive. Design restrictions of more complex bioorthogonal probes are mainly based on limited synthetic accessibility.

We have developed a fast and easily manageable strategy for the synthesis of complex multi-functional bioorthogonal probes that allow application in multiple coordinated experiments. Using established principles of solid-phase synthesis, we combine several functional building blocks (e.g., fluorophores, cross linkers, cleavable linkers, affinity tags etc.) to generate a platform of multimodal bioorthogonal probes; so-called MULTIMO-tags. The probe design contains a core motif that can be isotopically coded (e.g., D_2, ^{13}C, ^{15}N) as one of the flexible adaptable units. This allows equipping every probe with an inherent isotopic label, either for intact targeted glycoproteomics or for quantitative MS-analysis. Our synthesis strategy further allows probe release from the solid support and C-terminal functionalization in one step providing high flexibility regarding the bioorthogonal handles that can be introduced in the final synthesis step. The highly efficient synthesis strategy takes as little as three days to generate ready-to-use stocks of customized probes. Probes of the current generation contain three to six functional units and allow a combination of different imaging applications (fluorescence microscopy, plate reader, WB) followed by enrichment of tagged conjugates from complex mixtures (cell lysates), cleavage of bulky fluorophores and subsequent in-depth characterization via glycoproteomic approaches. Here, we showcase the application of the tailor-made probes for tagging and multi-modal analysis of metabolically labelled glycoproteins (MULTIMO-tags∗G).

(P227) Precision Enrichment of α2,3-Sialylated Glycoproteins through Biorthogonal Derivatization

Hongxia Bai[1], Collin McDowell[1], Richard Drake[1]

[1]*Medical Universoty of South Carolina, Charleston, SC, US,*

Sialylated glycoproteins play a pivotal role as disease progression markers, and α2,3 linked sialic acids are known components of the Sialyl Lewis X and Sialyl Lewis A tumor antigens. Due to multiple isomers and stability issues, accurate characterization of sialylation has been historically a challenge. While enrichment techniques exist, specificity for certain sialylated glycoconjugate isomers is lacking. We introduce a biorthogonal derivatization approach targeting glycoproteins with α2,3-sialylation for glycoproteomic analysis. Our approach is based on the use of a bioorthogonal strategy that leverages the differential amidation reactions characteristic to sialic acid residues: α2,6-linked sialic acids predominantly undergo dimethyl amidation, and α2,3 forms a bicyclic lactone. This intermolecular lactone of α2,3 sialic acids can be modified using an amine-azide, enabling subsequent conjugation to magnetic alkyne beads via click chemistry for magnetic capture. This approach was used with cultured pancreatic cancer cell lines and prostate cancer tissues.

In a 40-minute LC-MS acquisition of trypsin-digested pancreatic cancer cell lysates, 4,800 proteins were identified without enrichment. Using the alkyne bead enrichment, the number of detected proteins decreased to 1,700. Only 5% of these proteins were determined to be due to nonspecific binding, as seen from un-derivatized but enriched samples. This process highlighted glycoprotein carriers for α2,3-linked sialylated glycans, including CD44 antigen. For prostate FFPE tissues, prior mass spectrometry imaging indicated specific α2,3 sialylation to be present as tri- and tetraantennary N-glycans in tumors, and predominantly bi-antennary N-glycans for non-tumor regions. Upon comparison of tumor and normal regions using the present procedure, post-enrichment counts were 1,875 and 1,287 proteins, down from 3,607 and 3,537, respectively in non-enriched samples. Unique protein counts were 212 for tumors and 500 for normal regions, indicating enrichment rates of 43% and 36% for normal and tumor samples, respectively. Further verification was achieved with the direct analysis of peptides released from the beads following PNGase deglycosylation, verifying the potential sialylation sites of 77 and 61 proteins from normal and tumor regions.

In summary, we present a glycoproteomic mass spectrometry-based sequencing method targeting α2,3-sialylated glycoproteins. Our approach refines protein identification and reduces nonspecific binding. Poised for application to urine and serum, this technique offers potential in clinical and research settings, emphasizing the role of sialylated glycoproteins in disease.

(P228) Novel Sialic Acid Linkage-Specific *O*-Linked Glycan Analysis Method via Ester-to-Amide Derivatization

Hisatoshi Hanamatsu[1], Ikuko Yokota[1], Nobuaki Miura[1], Jun-ichi Furukawa[1,2]

[1]*Institute for Glyco-core Research (iGCORE), Nagoya University, Nagoya, Japan,* [2]*Department of Orthopaedic Surgery, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Hokkaido, Japan*

Glycosylation is one of the most common and complex post translational modifications of proteins. Glycans linked to serine or threonine residues are categorized as O-linked glycan. The O-glycosylation of proteins is involved in various biological

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processes, including cell-cell interactions, signal transduction, pathogenic infections, and cancer progression. Characterization of intact *O*-glycans has mostly been achieved using various chemical reaction approaches because there are no known *O*-glycan-specific endoglycosidases. However, there are several issues such as low yield of released *O*-glycans and significant peeling. Recently, a novel non-reductive β-elimination was reported for preparing intact *O*-glycans using various bases in the presence of hydroxylamine (NH_2OH) with lower peeling rate.

Many *O*-glycans are generally modified with sialic acid residues at their non-reducing termini through various linkages. Several unique sialic acid linkage-specific derivatization methods have been developed to distinguish sialic acid linkages by mass spectrometry. Recently, we also developed sialic acid linkage-specific alkylamidation of *N*- and GSL-glycans via lactone ring-opening aminolysis (Aminolysis-SALSA) or sialic acid linkage-specific ester-to-amide derivatization (LEAD-SALSA); however, these derivatizations have not been applied for *O*-linked glycan analysis.

In the present study, we assessed efficient non-reductive β-elimination in the presence of NH_2OH in combination with sialic acid linkage-specific alkylamidation for *O*-glycomic analysis. We established a simplified purification process for non-reductive *O*-glycans by glycoblotting combined with β-elimination in the presence of NH_2OH. Sialic acid linkage-specific derivatization successfully proceeded from ethyl-esterified *O*-glycans by lactone-driven ester-to-amide conversion. Furthermore, when simply combined with PNGase F digestion, simultaneous, quantitative, and sialic acid linkage-specific analysis of *N*- and *O*-glycans was accomplished.

(P229) Glycosylation of full-length antibodies in bacteria equipped with single-subunit oligosaccharyltransferase from *Desulfovibrio marinus*

Belen Sotomayor[1], May N. Taw[2], Thomas Connor Donahue[3], Sophia W. Hulbert[4], Parastoo Azadi[5], Lai-Xi Wang[3], Matthew P. DeLisa[1,6,*]

[1]Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, [2]Department of Microbiology, Cornell University, [3]Department of Chemistry and Biochemistry, University of Maryland, [4]Biochemistry, Molecular and Cell Biology, Cornell University, [5]Complex Carbohydrate Research Center, University of Georgia, Athens, [6]Cornell Institute of Biotechnology, Cornell University,

Asparagine-linked (*N*-linked) glycosylation is a complex and abundant post-translational modification commonly found in therapeutic proteins. These proteins, including monoclonal antibodies (mAbs) and fragment crystallizable (Fc) domain fusion proteins, contain glycan structures that influence the structure and functional properties of the therapeutic drug. Typically, glycoproteins are produced in mammalian cell lines capable of achieving human-like *N*- glycosylation. As an alternative, new protein glycosylation systems have arisen to understand, control, and customize the production of glycoproteins. In bacteria, the glycosylation machinery from *Campylobacter jejuni* has been successfully transferred to *Escherichia coli*, including the bacterial oligosaccharyltransferase (OST) PglB responsible for transferring the *N*-glycan from lipid-linked oligosaccharide (LLO) donors to asparagine residues of an acceptor protein. However, the replication of eukaryotic-like glycosylation in bacteria continues to be a significant challenge. One bottleneck is the extended sequon (D/E-X_{-1}-N-X_{+1}-S/T where X $\neq$ P) required by the archetypal OST, PglB from *C. jejuni*, which renders this enzyme incompatible with glycoproteins such as mAbs that lack this more specific acceptor sequence. To address this challenge, we previously mined the genomes of diverse bacterial species for PglB homologs with relaxed sequon specificity. While several enzymes with relaxed sequon specificity were uncovered, most notably PglB from *D. gigas*, these enzymes exhibited low efficiency (<2%) against the QYNST sequon of a human Fc domain. Here, we hypothesized that PglB homologs from other *Desulfovibrio* species might exhibit increased catalytic efficiency for non-extended sequons, thereby enabling glycosylation of broad substrates including human IgGs. To test this hypothesis, we assembled a collection of 19 OST candidates from *Desulfovibrio* spp. Among them, *Desulfovibrio marinus* (*Dm*PglB), was the only enzyme that could efficiently glycosylate any sequon in multiple acceptor protein targets regardless of the residue at the -2-position. Remarkably, *Dm*PglB was able to transfer bacterial *N*-glycans to the native N297 site of the Fc domain of full-length human IgG antibody expressed in *E. coli*. To generate uniform, authentic eukaryotic *N*-glycans, the bacterial glycans attached to the Fc were trimmed and subsequently remodeled *in vitro* by enzymatic transglycosylation, creating a recombinant IgG product bearing an asialo complex-type biantennary *N*-glycan (G2). This glycan-remodeled Fc engaged the FcγRIIIa receptor with the expected affinity while the Fc dimer containing the bacterial glycan did not, establishing that this glycoengineered product retained its effector functions. Taken together, we discovered a novel bacterial enzyme, *Dm*PglB, with previously unavailable biocatalytic capabilities that greatly expands the glycoengineering toolkit, especially in the generation of humanized IgG antibodies.

(P230) Identifying genetic regulators of cell-surface glycosylation using CRISPR genomic screening

Simon Wisnovsky[1]

[1]Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, V6T 1Z3.,

Unlike proteins, glycan sequences are not directly templated by a genetic code. Instead, they are produced through complex interactions between many different gene products. This complexity has historically impeded efforts to characterize glycan

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function in disease. CRISPR screening technology, however, has now enabled high-throughput manipulation of the mammalian genome and precise dissection of genetically convoluted cellular phenotypes. In prior work, we developed a genome-wide CRISPR screening method for identifying genetic pathways that regulate glycosylation in cancer cells. We applied this platform to generate a "genomic atlas" of genes that drive expression of ligands for the Siglec family of inhibitory immune receptors. Siglecs are signaling molecules that repress immune function by binding to specific glycans on cancer cells. They are thus emerging as key targets of interest for cancer immunotherapy. In this talk, I will summarize how our lab has been using genomic screening to identify and characterize novel regulators of Siglec ligand expression in acute myeloid leukemia (AML). Firstly, I will discuss in-depth functional characterization of a key glycosyltransferase enzyme that mediates biosynthesis of Siglec-9 ligands in a range of AML cell lines. Secondly, I will describe our discovery of an AML oncogene that broadly regulates biosynthesis of sialoglycans and our efforts to target this protein with small molecule inhibitors. Finally, I will briefly discuss our ongoing development of new glyco-genomic screening methods based on CRISPR activation (CRISPRa) technology. Taken together, this presentation will thus serve as a high-level introduction to the ways that CRISPR genomic screening can be usefully applied to the study of cancer glycobiology.

(P231) Efficient bacterial production of mucin-type O-glycoproteins

Nicole K. Thompson[1], Lyann Sim[2], Stephen G. Withers[2], Warren W. Wakarchuk[1]

[1]Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada, [2]Department of Chemistry, University of British Columbia, Vancouver, BC, Canada

We have developed an *Escherichia coli* expression platform with a customizable dual-plasmid system for the heterologous expression of proteins with mucin-type O-glycans. In combination with genomic integration of the *neuCAB* operon from *Neisseria meningitidis* for CMP-Neu5Ac biosynthesis, we have used this platform to demonstrate high-yielding production of authentically sialylated human interferon α-2b. Towards the aim of long-acting biopharmaceutical development where native O-glycosylation sites are not present, we are further investigating a variety of mucin-like domains as fusion partners, and show here that proline- and threonine-rich tandem repeats derived from MUC2 and the IgA1 hinge region are readily glycosylated by the human GalNAc-T2 isoform. Given the growing prevalence of pre-existing antibodies against polyethylene glycol in the general population, we ultimately aim to show the versatility of mucin-like domains as a non-immunogenic alternative for producing protein therapeutics with extended serum half-life by leveraging the natural protective function of sialoglycans as a post-translational modification.

(P232) Deglycosylated RBD produced in *Pichia pastoris* as a COVID-19 diagnosis tool and a vaccine candidate

Tommy Idrovo-Hidalgo[1], María F. Pignataro[1,2], Luis M. Bredeston[1,2], Fernanda Elias[3], María G. Herrera[1], María F. Pavan[1], Sabrina Foscaldi[1], Mayra Suireszcz[1], Natalia B. Fernández[1], Diana E. Wetzler[1,2], Carlos H. Paván[1,2], Patricio O. Craig[1,2], Ernesto A. Roman[1,2], Lucas A.M. Ruberto[1,2,4], Diego G. Noseda[2,5], Lorena I. Ibañez[1,2], Cecilia Czibener[2,5], Juan E. Ugalde[2,5], Argentinian AntiCovid Consortium[†], Alejandro D. Nadra[1,2], Javier Santos[1,2,*], Cecilia D'Alessio[1,2,*]

[1]Universidad de Buenos Aires, Buenos Aires, Argentina, [2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290 C1425FQB, Buenos Aires, Argentina, [3]Instituto de Ciencia y Tecnología Dr. César Milstein (Consejo Nacional de Investigaciones Científicas y Técnicas-Fundación Pablo Cassará), Saladillo 2468 C1440FFX, Buenos Aires, Argentina, [4]Instituto Antártico Argentino, Ministerio de Relaciones Exteriores y Culto, Buenos Aires, Argentina, [5]Universidad Nacional de San Martín, San Martín, Buenos Aires, Argentina,

[†]Argentinian Anticovid Consortium: Blaustein M, Bredeston L, Craig PO, D'Alessio C, Elías F, Fernandez NB, Gudesblat G, Herrera MG, Ibañez LI, Idrovo-Hidalgo T, Nadra AD, Noseda DG, Pavan C, Pignataro MF, Roman E, Ruberto L, Rubinstein N, Santos J, Wetzler D, Zelada A

During the COVID-19 pandemic various tools including protein-based vaccines were developed with varying degrees of effectiveness. The yeast *Pichia pastoris* is known to be a cost-effective and scalable eukaryotic system for producing recombinant proteins. It offers the advantages of an efficient secretion system and assistance in protein folding through the eukaryotic cell's secretory pathway. In a prior study, we expressed the Receptor Binding Domain (RBD) of the SARS-CoV-2 Spike protein in both *P. pastoris* and human cells. Although there were differences in size and glycosylation patterns, the structural and conformational characteristics of the proteins were remarkably similar. However, we addressed potential issues related to yeast-added high mannose glycan moieties, which could trigger non-specific immune responses, by deglycosylating the RBD under native conditions. This resulted in a highly pure, uniform, and well-folded monomeric protein with circular dichroism spectra matching those of glycosylated RBD and RBD produced in human cells. The deglycosylated RBD was obtained in a single-step process, yielding high quantities, and it effectively differentiated between sera from SARS-CoV-2-negative and -positive patients.

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Furthermore, when used as an immunogen, the deglycosylated variant elicited a humoral immune response stronger than the glycosylated RBD, produced antibodies with greater neutralizing capacity, and induced a better cellular response. This approach can be employed to cost-effectively produce numerous antigens that require glycosylation for proper folding and expression but not for recognition purposes.

(P233) Identification of a Dominant Negative Regulator of HS3ST1 Enzyme Inhibiting Heparan Sulfate Sulfation

Vivien Uyen Doan[1], Andrea Kaminski[2], Yongmei Xu[1], Zhangjie Wang[1], Lars Pedersen[2], Jian Liu[1]

[1]*Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA,* [2]*Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC, USA*

Heparan sulfate (HS) is a complex polysaccharide that plays critical roles in numerous biological processes. HS3ST1 is an essential enzyme in the biosynthesis of HS, responsible for the initial 3-O-sulfation step that is required for further modification of HS. In this study, we identified a novel splice variant of HS3ST1, which carries a missense mutation (E86Q) and acts as a dominant negative regulator of HS sulfation.

We demonstrate that the mutant HS3ST1 variant (HS3ST1-E86Q) inhibits the activity of wild-type HS3ST1 in vitro and in vivo, leading to a significant reduction in HS sulfation. Overexpression of HS3ST1-E86Q in cultured cells reduces the level of 3-O-sulfated HS chains and impairs their biological activity. We speculate that the presence of this variant may contribute to the regulation of HS sulfation in these tissues and impact their function.

Overall, our study reveals a novel splice variant of HS3ST1 with a missense mutation that acts as a dominant negative regulator of HS sulfation. This finding highlights the importance of alternative splicing and genetic variations in the regulation of HS biosynthesis and its impact on biological processes. Understanding the molecular mechanisms underlying altered HS sulfation patterns may provide insights into the pathogenesis of various diseases, including cancer and neurodegenerative disorders. Furthermore, our study highlights the potential of splice variants as a novel therapeutic target for the treatment of HS-related disorders. Specifically, the development of drugs targeting the inhibition of dominant negative HS3ST1 variants may provide a promising avenue for the treatment of diseases associated with HS sulfation defects.

(P234) Engineering IgA proteases to target glycoform-specific IgA1

Angel Kuo[1], Brendan Floyd[1], Carolyn Bertozzi[1,2]

[1]*Department of Chemistry, Stanford University, Stanford, CA 94305, USA,* [2]*Howard Hughes Medical Institute, Stanford, CA 94305, USA*

O-glycosylation of cell surface and secreted glycoproteins is well-known to play important roles in homeostasis and disease progression. Mucins are a class of heavily O-glycosylated proteins that have been extensively studied in the context of cancer. In addition to the canonical mucins often studied in cancer and gut health, recent work has shown other mucin-domains glycoproteins to have roles in human diseases. One such example is the mucin-like domain of IgA1. IgA1, which is present in the bloodstream and in mucosal membranes, has a hinge region bearing several O-glycans. The IgA1 mucin-like domain is known to have a role in the pathogenesis of IgA nephropathy. IgA nephropathy is a renal disease that leads to impaired kidney function and is thought to be caused by renal deposits of under-galactosylated IgA1. To date, there have been no reported ways to target and degrade this disease-specific glycoform of IgA1.

Here we report work towards a strategy to selectively degrade the disease-relevant glycoforms of IgA1. Previous work characterized bacterial proteases that are incredibly specific for human IgA1. Other recent work characterized bacterial proteases that selectively cleave mucins in their mucin-domains. These mucin-selective proteases, coined mucinases, have a range of specificity for certain glycoforms, some accommodating all types of glycoforms and others only tolerating specific glycoforms. Through structural modeling, protein engineering, and biochemical assays, we developed fusion proteases comprising of two parts: an IgA protease domain to target IgA1, and a mucinase domain that is non-specific for IgA1 but selectively cleaves particular glycoforms of the IgA1 mucin-like domain. We demonstrate efforts towards this method, which will enable us to study IgA1 glycoforms in different diseases and selectively degrade IgA1 glycoforms of interest.

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(P235) Human MUC2 can be non-invasively acquired from fecal material for visualization and functional O-glycomics in health and disease

Noah Fancy[1, 2], Darrek Kniffen[1], Mackenzie Melvin[1], Negin Kazemian[3], Javad Sadeghi[3], Clara A. Letef[1], Leah D'Aloisio[1], Amanda G. Copp[1], Kris Chadee[4], Sanjoy Ghosh[1], Deanna L. Gibson[1], Sepideh Pakpour[3], Wesley F. Zandberg[2], Kirk Bergstrom[1]

[1]*University of British Columbia (Okanagan), Department of Biology, Kelowna, British Columbia, Canada,* [2]*University of British Columbia (Okanagan), Department of Chemistry,* [3]*University of British Columbia (Okanagan), School of Engineering,* [4]*University of Calgary, Cumming School of Medicine, Calgary, Canada,*

The *O*-glycoprotein Mucin-2 (MUC2) forms the protective colon mucus gel. While animal models have demonstrated the importance of orthologous Muc2 and its *O*-linked oligosaccharides, few studies have explored human MUC2 in similar depth. Recent studies have revealed secreted MUC2 is bound to human feces. We therefore hypothesized human fecal MUC2 (HF-MUC2) was accessible for visualization, purification and downstream structural and functional characterization. We tested this via histologic and quantitative imaging on Carnoy's-fixed human fecal sections; extraction from feces for proteomic and *O*-glycomic characterization; and functional studies via growth and metabolic assays in vitro. Quantitative imaging of solid fecal sections showed a continuous mucus layer of varying thickness along human fecal sections with barrier functions intact. However, barrier function was lost on loose samples. Lectin profiling showed HF-MUC2 bound several lectins but was absent for *Ulex europaeus 1* (α1,2 fucose binding) and *Sambucus nigra* agglutinin (α2,6 Sia binding), and did not have obvious b1/b2 barrier layers. HF-MUC2 separated by composite urea agarose polyacrylamide gel electrophoresis (UreaAgPAGE) showed high molecular weight glycoprotein bands (~1–2 MDa). Proteomics and western analysis confirmed enrichment of MUC2 and potential MUC2-associated proteins in HF-MUC2 extracts. HF-MUC2 *O*-glycomics revealed extensive complex *O*-glycosylation characterized by abundant fucosylation, moderate sialylation, and little sulfation vs. porcine colonic MUC2 and murine fecal Muc2. *O*-glycans were functional and supported the growth of *Bacteroides thetaiotaomicron* and short chain fatty acid (SCFA) production in vitro. MUC2 could be similarly analyzed from ulcerative colitis stools, which demonstrated a similar glycosylated high molecular weight MUC2 band, but an altered glycomic profile and differential growth and SCFA production vs. healthy samples. These studies describe a new non-invasive platform for human MUC2 visualization and functional glycomics in health and disease.

(P236) Development of a new glycan-related pathway repository: GlycoPathwayRepo

Sunmyoung Lee[1], Yushi Takahashi[1], Tamiko Ono[1,2], Masaaki Shiota[1,2], Akihiro Fujita[1,2], Kiyoko F. Aoki-Kinoshita[1,2]

[1]*Graduate School of Science and Engineering,* [2]*Soka University Glycan and Life Systems Integration Center (GaLSIC), Soka University*

Glycosylation has played critical roles in a wide range of cellular processes, from binding ligands to receptors on the cellular surface of signal transduction pathways to determining the fate of proteins encoded by the genome. Incorporating glycan information, such as glycan structure and glycan binding site data, into the form of pathways provided by pathway analysis, which is predominantly derived from gene and protein resources, enables users to gain precise knowledge and insight into the biological pathway. We have developed a repository named GlycoPathwayRepo to provide a user-friendly interface and repository for submitting pathway data that reflects information on glycosylation in the relevant pathway. GlycoPathwayRepo is designed to collect pathway data containing standardized information such as species, tissue, cell type, modification on proteins or lipids, etc., depending on user input. It has two modes for inputting the glycan-related pathway data: glycan biosynthesis pathway and glycan-related pathway. The former mode focuses on the biosynthesis of glycan structures and glycoenzymes, while the latter mode focuses on glycan modifications of proteins and lipids, where users can simply input their pathway data by selecting an entity from a list provided by the system. After input is completed, users can review each registered pathway data as a figure. Also, to share the acquired repository data with other pathway data, Semantic Web Techniques, which are designed to connect data semantically to make it easier for machines to interpret the data on the Web, is used. The collected data will contribute to the creation of a comprehensive database to reveal the function of glycans in normal and disease pathways. We expect that our repository will contribute to accelerating data sharing of glycobiology with other omics data by providing tools where wet-lab researchers can share their meaningful data in a formalized and exchangeable format.

(P237) Study of Glycan Effect on Protein-Protein Interaction by Using Artificial Small Glycoproteins

Harumi Nishikawa[1], Ryo Okamoto[1,2], Yuta Maki[1,2], Yasuhiro Kajihara[1,2]

[1]*Grad. Sch. Sci., Osaka Univ.,* [2]*FRC, Grad. Sch. Sci., Osaka Univ.*

Functional analysis of homogeneous glycoproteins prepared by chemical synthesis is a powerful approach to elucidate the functions of glycans of glycoproteins at a molecular level. Recently, we found that the hydration of glycans on the protein

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surface may affect the binding affinity of protein-protein interaction. Herein we carried out the synthesis of a small glycoprotein as a new probe to clarify the potential functions of glycan-hydration. We selected a small protein that specifically binds to the Fc region of IgG antibodies as a scaffold of the glycoprotein. We have successfully synthesized a small glycoprotein having a biantennary N-glycan by using Fmoc solid-phase synthesis. We found that the glycan changes the binding affinity between the small protein and the Fc region of IgG. The details of the synthesis of the new glycoprotein and analysis of binding activity will be discussed in this presentation.

(P238) Glydentify, a tool for unsupervised classification of glycosyltransferase function

Aarya Venkat[1], Natarajan Kannan[1]

[1]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA,*

Protein language models have emerged as a powerful tool for predicting protein function by capturing the underlying grammar and syntax of protein sequences. Here, we introduce Glydentify, an open-source and user-friendly application designed for the unsupervised classification of glycosyltransferases (GTs). Utilizing the state-of-the-art ESM2 protein language model, Glydentify extracts high-dimensional sequence embeddings to accurately classify GTs into fold A families with 92% confidence. The tool also predicts GT-A donor binding preferences with an accuracy of 87%. Notably, Glydentify identifies key residues that contribute to a prediction, thereby adding an explainable AI component to the application. With an intuitive interface powered by Gradio, Glydentify requires no programming experience from the user, democratizing access to cutting-edge deep learning technologies for glycosyltransferase research. The application is freely available on GitHub and can be accessed directly through any web browser.

This study was supported by funding from the NIH from R35 GM139656 (N.K.) and from ARCS foundation scholarship (A.V.).

(P239) Screening of the human gut microbiome reveals an alternative glycan degradation pathway of unprecedented substrate breadth

Seyed Amirhossein Nasseri[1], Aleksander Lazarski[1], Imke Lemmer[1], Chloe Zhang[1], Eva Brencher[1], Hong-Ming Chen[1], Leo Betschart[1], Liam Worrall[1], Natalie C.J. Strynadka[1], Stephen G. Withers[1]

[1]*Departments of Chemistry and Biochemistry, University of British Columbia,*

Most glycosidases characterised to date hydrolyse glycosidic bonds using one of the “Koshland” mechanisms. By screening a human gut microbiome metagenomic library with an assay designed to selectively identify enzymes that do not use such Koshland mechanisms we identified a commonly occurring gene cluster encoding enzymes of exceptionally broad substrate specificity. We then characterised their mechanisms and determined their structures. These same enzymes break both alpha- and beta- glycosidic bonds with no concern for connectivity between sugars. They also digest substrates that are not cleaved by standard glycosidases, including thioglycosides such as glucosinolates and pseudo-glycosidic bonds of pharmaceuticals such as acarbose. The enzymes employ a distinct mechanism that involves stepwise oxidation, elimination and hydration steps. These are separately catalysed by enzyme modules that are often interchangeable between organisms and substrate classes. While there are hints in the literature, this alternative pathway for glycan degradation has remained largely unrecognised and is of unprecedentedly broad specificity.

(P240) Impact of brain tissue fixation and processing on perineuronal net analyses in mice and non-human primates

Jaden Le[1], Aarun Hendrickson[1], Ingrid Redford[2], Emily Quah[2], Caeley Bryan[2], Bao Anh Phan[2], Asmit Kumar[1], Jarrad Scarlett[2,3], David Tovar[3], Kimberly Alonge[1,2]

[1]*Department of Medicinal Chemistry, University of Washington, Seattle, WA,* [2]*Department of Medicine, University of Washington, Seattle, WA,* [3]*Department of Pediatric Gastroenterology and Hepatology, Seattle Children’s Hospital, Seattle, WA, USA,*

Perineuronal nets are extracellular matrix structures comprised of chondroitin and dermatan sulfate-glycosaminoglycans (CS/DS-GAGs). Histological imaging of brain PNNs is achieved using *Wisteria floribunda* agglutinin (WFA) labeling of PNN CS/DS-GAGs, while composition can be determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although these methods are used to determine PNN CS/DS-GAG abundance and composition, it’s unknown whether brain fixation or processing influence these outcomes.

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We first explored whether tissue processing, using cryosectioning (CRYO) or paraffin embedding (PE), influence PNN analyses. Ten mice (5M/5F) were perfused with PBS and post-fixed in 4% paraformaldehyde (PFA). Brains were cut sagittal, and one hemisphere was prepared as 30-um free floating tissues (CRYO technique) and the second hemisphere processed as 5-um direct mounted tissues (PE technique). Histochemical analysis show a 78.9% reduction ($p<0.0001$) in hippocampal WFA+ PNNs in the PE processed hemisphere compared to CRYO processed side. LC-MS/MS analysis of hippocampal CS/DS isomers also show baseline differences ($p<0.0001$): PE processed tissues exhibit higher 4S-CS (CRYO: 67.8%, PE: 90.4%) and 4S6S-CS (CRYO: 0.3%, PE: 0.8%) isomers, while CRYO prepared tissues exhibit higher 0S-CS (CRYO: 14.4%, PE: 2.7%), 6S-CS (CRYO: 12.8%, PE: 4.7%), 2S6S-CS (CRYO: 3.3%, PE: 0.9%), and 2S4S-DS (CRYO: 1.4%, PE: 0.5%) isomers. In a second cohort of mice, we determined that fixative (4% PFA vs 10% formalin) did not influence hippocampal WFA ($p=0.09$) or CS/DS isomers ($p=0.25$) between groups, suggesting tissue processing (not fixative) influences PNN analyses.

We then explored whether we could correct for these CS/DS baseline differences. By comparing CS/DS isomers isolated from CRYO vs PE processed tissues within each mouse, we discovered reproducible correction factors for each isomer: 0S-CS (0.19), 4S-CS (1.34), 6S-CS (0.37), 4S6S-CS (2.29), 2S6S-CS (0.26), and 2S4S-DS (0.39). Adjusting the CRYO group using these factors normalizes baseline compositional differences between CRYO and PF groups ($p=0.95$). To determine translational relevance, we compared hippocampal CS/DS isomers between three CRYO vs PE prepared non-human primate (*M. nemestrina*) tissues and observe similar baseline CS/DS differences ($p=0.009$): NHP hippocampal PE processed tissues exhibit higher 4S-CS (CRYO: 53.6%, PE: 78.4%) and 4S6S-CS (CRYO: 1.2%, PE: 2.2%) isomers, while CRYO processed tissues exhibit higher 0S-CS (CRYO: 24.2%, PE: 9.3%), 6S-CS (CRYO: 10.4%, PE: 5.9%), and 2S6S-CS (CRYO: 3.3%, PE: 2.4%) isomers. Adjusting the CRYO prepared group using corrections factors normalizes the baseline composition between CRYO and PF groups ($p=0.95$). These results provide strong, translational evidence that tissue processing greatly influences both PNN glycan histology and composition analyses, and that corrections must be made to account for baseline differences before comparing groups.

(P241) Enzymatic strategies for the synthesis of vaccine antigens from Gram-negative pathogens

Javier O. Cifuente[1,2,†], Julia Schulze[3,†], Andrea Bethe[3,†], Valerio Di Domenico[1,†], Christa Litschko[3], Insa Budde[3], Lukas Eidenberger[4], Hauke Thiesler[3], Isabel Ramón Roth[3], Monika Berger[3], Heike Claus[5], Cecilia D'Angelo[1,2], Alberto Marina[2], Rita Gerardy-Schahn[3], Mario Schubert[6], Marcelo E. Guerin[1,2,7], Timm Fiebig[3]

[1]*Structural Glycobiology Laboratory, Biocruces Bizkaia Health Research Institute, Cruces University Hospital, 48903 Barakaldo, Bizkaia, Spain,* [2]*Structural Glycobiology Laboratory, Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Building 801A, 48160 Derio, Spain,* [3]*Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany,* [4]*Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, 1190 Vienna, Austria,* [5]*Institute for Hygiene and Microbiology, University of Würzburg, Würzburg, Germany,* [6]*Department of Biosciences, University of Salzburg, Salzburg, Austria,* [7]*Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain,*

[†]These authors contributed equally

Capsular polysaccharides (CPS) are important virulence factors that protect bacterial pathogens from the host immune system. They are structurally diverse and can consist exclusively of saccharide units, or sugars alternating with phosphate or polyol-phosphate moieties. CPS can be used as antigens in highly effective glycoconjugate vaccines, in which they are coupled to a carrier protein to induce a T-cell dependent immune response. The manufacturing of glycoconjugate vaccines includes the purification of CPS from pathogen culture. To reduce biohazard and costs, chemical and enzymatic synthesis have been extensively studied as alternatives for CPS production.

Our research focusses on the biochemical and structural characterization of capsule biosynthesis enzymes and the development of enzyme-based synthesis cascades for the provision of CPS. The enzymes required for these protocols include (i) nucleotide-sugar epimerases and nucleotidyltransferases to generate substrates like nucleotide sugars and polyols, (ii) capsule polymerases using said substrates to polymerize the CPS, and (iii) CPS-modifying transferases that add glycosyl- or O-acetyl groups to e.g. distinguish between serotypes.

Here, we present the biochemical and mechanistic characterization as well as the biotechnological exploitation of capsule biosynthesis enzymes required for the generation and modification of phosphate-containing CPS from the Gram-negative pathogens *Neisseria meningitidis*[1], *Haemophilus influenzae*[2,3] and *Actinobacillus pleuropneumoniae*[4,5]. Special focus will be laid on Bcs3, the capsule polymerase from *Haemophilus influenzae* serotype b (Hib). Bcs3 is a multi-enzyme machine, in which a ribofuranosyltransferase, a phosphatase and a ribitol-phosphate transferase work in concert during the assembly of the complex Hib polymer, which is tethered to the enzyme by a polymer-binding domain. Bcs3 forms a basket-like dimer harboring two active centers and creating a protected environment for the parallel synthesis of two Hib capsule polymer chains. An enzyme-based cascade was established for the generation of the two substrates of Bcs3, CDP-ribitol and 5-phospho-α-D-ribose-1-diphosphate (PRPP), allowing Hib polymer synthesis to start from widely available ribose-5-phosphate.

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This study was funded by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)— project number 412824531.

(P242) Protein-O-Mannosylation By Non-Sec/Tat Secretion Translocons In Actinobacteria

Hirak Saxena[1], Rucha Patel[1], John Kelly[2], Warren Wakarchuk[1]

[1]*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada,* [2]*Human Health Therapeutics, National Research Council of Canada, Ottawa, Ontario, Canada*

Protein-O-mannosylation (POM) is a form of O-glycosylation that is ubiquitous throughout all domains of life and has been extensively characterized in eukaryotic systems. However, in prokaryotes this process has only been investigated in terms of pathogenicity (in *Mycobacterium tuberculosis*) even though there are many non-pathogenic bacteria that are known to regularly carry out POM. To date, there is no consensus on what benefit POM imparts to the non-pathogenic bacteria that can perform it. Though the generation of a POM deficient mutant of *Corynebacterium glutamicum* – a widely utilized and known mannosylating actinobacteria – this work shows that even closely related actinobacterial GT-39s (the enzymes responsible for the initiation of POM) can potentially have different activities and substrate specificities for targets of POM. Moreover, presented here is evidence that POM does not only occur in a SEC-dependent manner; POM also occurs with TAT and non-SEC secreted substrates in a specific and likely tightly regulated manner. Together these results highlight the need for further biochemical characterization of POM in these and other bacterial species to help elucidate the true nature of its biological functions.

(P243) Sulfation Signatures of Chondroitin Sulfate Proteoglycans Unveiled Through Mass Spectrometry and Nanopore Sequencing

Xisheng Zhou[1], Fengying Tang[2], Charles W. Frevert[2], Jeff Nivala[3], Kimberly Alonge[4,5]

[1]*Department of Chemistry, University of Washington, Seattle, WA,* [2]*Department of Comparative Medicine, University of Washington, Seattle, WA,* [3]*Department of Computer Science and Engineering, University of Washington, Seattle, WA,* [4]*Department of Medicinal Chemistry, University of Washington, Seattle, WA,* [5]*Department of Medicine, University of Washington, Seattle, WA,*

Chondroitin sulfate proteoglycans (CSPGs), which are comprised of a CS core protein and chondroitin and dermatan sulfate-glycosaminoglycan (CS/DS-GAG) attachments, play a key role in neurodevelopment and circuity plasticity during postnatal development. The CS/DS disaccharide unit can be non- (0S-CS), mono-(4S-CS and 6S-CS), or di-sulfated (4S6S-CS, 2S6S-CS, 2S4S-DS), and evidence suggests that CS/DS glycan motifs may function as molecular recognition elements for the attraction and binding of extracellular proteins associated with these developmental processes.

We first predicted that the expression of different CSPGs may express distinct CS/DS-GAG glycan attachments in a time- and region-specific manner. Here, we analyzed the composition of fibroblast CS/DS-GAGs using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our Preliminary data show that lower molecular weight CSPGs (LMW, Biglycan/Decorin) exhibit differential CS/DS isomer patterning compared to higher molecular weight CSPGs (HMW, Aggrecan/Versican) including 4S-CS (LMW: 63.9±1.7%, HMW: 29.8±1.3%, mean±SEM), 6S-CS (LMW: 22.9±1.1%, HMW: 47.4±1.0%), and 2S4S-DS (4S-CS (LMW: 0.5±0.1%, HMW: 0.04±0.02%). These observations provide support that 1) CS/DS glycans exhibit differential sulfation composition specific to the underlying core protein, and 2) a ‘sulfation code’ may exist along the CS/DS-GAG chain for extracellular protein binding.

Such sequencing concepts have common features with DNA, RNA, and proteins that use sequence-specific patterns to interact with target molecules, and nanopore sensor arrays have been used successfully to sequences these targets. We next sought to determine whether nanopore sensing could be applied to enable sequencing of sulfated CS/DS-GAGs at the single-molecule level. We first analyzed 30kDa 4S-CS, 6S-CS and 2S6S-DS glycan chains of homozygous composition using the commercially-available Oxford Nanopore Technology MinION high-throughput nanopore sensor array. Our Preliminary Data shows the MinION flow cell can detect the electrophoretically-driven translocation of CS and DS glycans through the nanopores, which resulted in temporary drops in ionic conductance when compared to buffer alone. These drops produced unique current magnitude (ΔI) and duration of time (dwell time, Δt) signatures for each CS and DS chain variety, and using machine learning to predict the 4S-CS and 6S-CS chains of interest, we established a 71.4% (4S-CS) and 83.6% (6S-CS) prediction accuracy for each glycan chain tested. A limiting factor of the MiniION nanopore is that voltage-driven translocation of the CS and DS chains are too fast to be accurately captured and transferred into single molecular sequencing data. In order to generate accurate single molecule sequencing data for each isomer such as 4S-CS and 6S-CS, we attached a streptavidin to biotin-labeled CS/DS glycan chains to block the pore entry and prevent the translocation of these glycans through the nanopore.

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(P244) Proteoglycan Mimetics for Probing Heparan Sulfate – Growth Factor Interactions

Julianna Follmar[1], Ryan N. Porell[2], Bryce Timm[1], Pravinkumar Choudhary[1], Alexander Parmele[3], Bryan E. Thacker[4], Charles A. Glass[4], Kamil Godula[1,5]

[1]*Department of Chemistry and Biochemistry, University of California, San Diego, CA, USA,* [2]*GlycoMIP, Virginia Tech, Blacksburg, VA, USA,* [3]*Department of Chemistry, University of California, Berkeley, CA, USA,* [4]*TEGA Therapeutics, Inc., San Diego, California, United States,* [5]*Glycobiology Research and Training Center, Departments of Medicine and Cellular and Molecular Medicine, University of California, San Diego, CA, USA,*

Proteoglycans (PGs) are essential components of the extracellular matrix, playing a pivotal role in regulating various biological functions, including binding, cell adhesion, migration, signaling, and differentiation. Among the classes of proteoglycans, Heparan Sulfate Proteoglycans (HSPGs) stand out as membrane-bound proteins capable of interacting with over 400 binding proteins. PGs feature linear polysaccharide chains modified with varying degrees of sulfation, organized into distinct domains that are responsible for protein binding. While the development of glycan microarrays has facilitated the profiling of ligand specificities for several HSPG-binding proteins, creating proteoglycan mimetics has proven to be a notably challenging endeavor. We have devised novel conjugation strategies to produce reagents known as neoProteoglycans (neoPGs). These reagents possess the unique ability to integrate full-length, biologically derived HS structures. The efficient and tunable strategy involves the use of a protein core combined with a reducing-end conjugation method to mimic the architectural characteristics of cellular PGs. The neoPG reagents enable effective and efficient probing of ligand binding interactions and offer insights into HS-regulated signaling within cells. The development of these innovative reagents represents a crucial advancement to our understanding of the intricate structure-function relationship of HSPGs.

(P245) Developing and Applying Intact Glycoproteomics Analysis Workflows for Cancer Diagnostics

Sharon Pitteri[1]

[1]*Stanford University,*

Despite the well-known fact that glycosylation is aberrant in cancer, the link between glycan and protein information remains relatively poorly understood due to the analytical challenges associated with studying the complex modification in biological samples. In recent years, mass spectrometry has emerged as a preeminent tool to study protein glycosylation, and has enabled the characterization of intact glycopeptides, thereby allowing retention of information about both the glycan and modified protein. We have developed intact glycoproteomic analysis workflows and applied them to a variety of human clinical samples including tissue, urine, and other fluids, to identify, quantify, and characterize sites of protein glycosylation in a systematic fashion. Our approach includes protein extraction, enzymatic digestion, and glycopeptide enrichment using strong anion exchange with electrostatic repulsion hydrophilic interaction chromatography prior to analysis by LC-MS/MS on an Orbitrap mass spectrometer. In a recent glycopeptide analysis of clear cell renal cell carcinoma tissue samples, we identified more than 120,000 unique high confidence glycopeptides from more than 2000 glycoproteins. Protein glycosites were classified by glycan subtype as either complex (with or without sialic acids and/or fucose), high mannose, or hybrid. The diversity of glycosylation was compared between cancer and patient-matched adjacent non-cancer kidney tissue. Notably increased heterogeneity in glycosylation was observed in cancer compared to non-cancer tissue for specific glyosites and proteins, yielding intriguing information about the biological pathways altered by glycosylation in cancer. Overall results and specific examples will be presented. Results from applying our workflows to pancreatic cyst fluid and prostate cancer urine samples will also be discussed.

(P246) Optimization of a multi-omic workflow for biomarker discovery in a medaka fish model of chronic low dose ionizing radiation exposure

Sydney Bedillion[1], Franklin E. Leach, III[2], Michael Tiemeyer[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA,* [2]*Department of Chemistry, University of Georgia, Athens, GA*

The effects of high dose ionizing radiation have been extensively characterized to include single and double stranded breaks in DNA, protein damage, metabolic complications, and severe risk for cancer. Human exposure to high dose ionizing radiation is relatively rare; however, exposure to low dose ionizing radiation (LDIR) is common through both medical and environmental sources. The impact of chronic LDIR is much less understood and has been difficult to assess without the identification of an informative set of biomarkers. The development of efficient and reproducible multi-omic workflows for proteomic, lipidomic, and metabolomic analyses alongside glycomic examination will facilitate the elucidation of LDIR specific biomarkers.

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We will expand on previous work completed by our group examining the proteomic and glycomic changes detected in medaka fish body regions and organ sets post LDIR exposure. Previous analysis identified 26 N-glycan structures and over 500 proteins that presented statistically significant differences when compared to control fish. The abundance of glycans containing fucose and/or sialic acids were most responsive to radiation exposure. Importantly, these terminal and branching glycan modifications are closely associated with inflammatory responses and may suggest an underlying target for intervening in low dose/chronic radiation responses. Meanwhile, proteomic examination revealed changes in fatty acid beta-oxidation and overall lipid metabolism all of which could be further explored for their potential biodosimetry applications. In our current work, previously established extraction protocols, taking advantage of readily available solvents, are applied to wild type medaka fish liver and brain tissue to determine suitability for multi-omic analysis. All extractions precipitate proteins by either a two-step extraction, one-phase extraction, or simultaneous extraction of polar metabolites and lipids. N-linked glycans will be isolated from wild type medaka fish liver and brain tissue to investigate how altered tissue metabolism leads to changes in lipid, protein, and glycan expression. Proteomic, lipidomic, metabolomic, and glycomic samples are analyzed by workflows optimized for specific molecular types and utilize various combinations of liquid chromatography and direct infusion tandem mass spectrometry on Orbitrap MS platforms. All extraction protocols yielded materials amenable to MS-based analysis. We will extend our optimized protocol to irradiated fish tissue that has been harvested as well-defined organs to gain a deeper understanding of the multi-omic changes that occur post exposure and to provide valuable target candidates for LDIR biomarkers.

(P247) A workshop on the functional impact of glycans and their curation

Karina Martinez[1], Jon Agirre[2], Yukie Akune[3], Kiyoko F. Aoki-Kinoshita[4], Cecilia Arighi[5], Kristian B. Axelsen[6], Evan Bolton[7], Emily Bordeleau[8], Patrice Duroux[9], Nathan J. Edwards[10], Elisa Fadda[11], Ten Feizi[3], Catherine Hayes[6], Callum M. Ives[11], Hiren J. Joshi[12], Krishna Prasad Khakurel[13], Sofia Kossida[9], Frederique Lisacek[6], Yan Liu[3], Thomas Lütteke[14], Adnan Malik[15], Taciana Manso[9], Maria Martin[15], Akul Y. Mehta[16], Sriram Neelamegham[17], Kalpana Panneerselvam[15], Rene Ranzinger[18], Sylvie Ricard-Blum[19], Gaoussou Sanou[9], Vijay Shanker[5], Paul D. Thomas[20], Michael Tiemeyer[18], James Urban[21], Rand Vita[22], Jeet Vora[1], Yasunori Yamamoto[23], Raja Mazumder[1]

[1]The George Washington University, [2]University of York, [3]Imperial College, [4]Soka University, [5]University of Delaware, [6]SIB Swiss Institute of Bioinformatics, [7]National Institutes of Health, [8]The University of British Columbia, [9]University of Montpellier, [10]Georgetown University, [11]Maynooth University, [12]University of Copenhagen, [13]The Extreme Light Infrastructure ERIC, [14]Justus-Liebig-University Gießen, [15]European Bioinformatics Institute, [16]Harvard Medical School, [17]University at Buffalo, State University of New York, [18]The University of Georgia, [19]University Lyon, [20]University of Southern California, [21]University of Gothenburg, [22]La Jolla Institute for Allergy & Immunology, [23]Research organization of Information and Systems,

Glycans impact many biological processes such as protein folding, immune response and molecular interactions. However, due to the complex and diverse nature of these biomolecules, the definition of glycan function has been elusive. A community effort is needed to agree on standards, annotations and functional terms to better represent the functional impact of glycans in bioinformatics resources worldwide. The workshop on glycan function curation was held in conjunction with the 16th Annual International Biocuration Conference in Padua, Italy in April 2023. Bioinformaticians, curators and glycobiologists from more than twenty international projects and resources shared insights on their current curation practices and data of interest. Discussions were guided by four questions: (i) How would your resource extract glycan-related data from literature or other sources? (ii) What type of glycan-related data would be useful to your resource, that you don't currently have access to? (iii) What type of data, that you do have, might enhance glycan-related data? (iv) What standards does your resource use for the glycan-related annotations? This resulted in the identification of needs/bottlenecks, potential solutions and opportunities to improve glycan-related annotations and increase data sharing amongst participating resources. The workshop laid the groundwork for future meetings and collaborations designed to further explore ideas such as co-curation of publications, enhancing text mining efforts, naming glycan functions and increasing glycoscience data connectivity with data from databases and knowledgebases in different fields of research. A second workshop is taking place in Kona, Hawaii prior to the 2023 Society for Glycobiology meeting and will bring together experts in glycan function and structure to produce a list of terms that can serve as a blueprint for bridging between glycan functions, gene/protein functions, and other large datasets.This poster will outline key outcomes from the April meeting and give an overview of progress towards achieving the primary goals of community participation in advancing glycan annotation and standards in biomedically-focused resources.

(P248) Antibody Array Based N-Glycan Imaging of Captured Immune Cells

James Dressman[1], Colin T. McDowell[1], Xiaowei Lu[1], Peggi M. Angel[1], Richard R. Drake[1], Anand Mehta[1]

[1]Department of Cell & Molecular Pharmacology, Medical University of South Carolina,

Cell surface N-glycosylation plays an important role in both the innate and adaptive immune response through the modulation of cell surface receptors. The study of immune cell N-glycosylation is increasingly becoming a field of interest, but is hindered by the

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complexity of cell type specific N-glycan analysis. Analytical techniques such as chromatography, LC-MS/MS and lectin staining all are currently used to analyze immune cell N-glycosylation. Pitfalls from each analytical technique impede either throughput or the acquisition of structural data, thereby reducing their feasibility for N-glycan study. Here, we report development of a rapid antibody array-based approach for the capture of specific nonadherent immune cells coupled with MALDI-IMS to analyze cell surface N-glycosylation, increasing the feasibility of immune cell N-glycan analysis. Cell N-glycosylation was analyzed using MALDI-IMS, relying on enzymatically released N-glycans from cells bound to immobilized antibodies. Glycans were released by peptide N-glycosidase (PNGase F PRIME) digestion coupled with the removal of terminal sialic acid residues via sialidase digestions or the stabilization and derivatization of the sialic acid residues by an amidation-amidation reaction. The primary goal of the project was to determine the feasibility of cell capture with an immobilized antibody specific to a surface receptor, and to analyze captured cell N-glycans via MALDI-IMS. Jurkat cells were initially captured with an anti-CD7 antibody coupled to a hydrogel coated slide. Following blocking and incubation, cells bound only to the antibody spot, immobilized by the cell receptor antibody interaction. Utilizing well established N-glycan MALDI imaging protocols, enzymatically released N-glycans were able to be detected from antibody captured cells. Following confirmation of capture, the method was further optimized to improve reproducibility. The intended application of this assay is to examine nonadherent immune cell N-glycosylation from heterogenous populations due to their inherent difficulty to analyze. The efficiency and robustness of the assay has been demonstrated with a PBMC cohort of 30 healthy men where we were able to capture and acquire N-glycan data from CD4, CD8 and B cells. Expansion of the immuno-array to other cell types is ongoing and the application of the methodology to clinical cohorts for biomarker discovery is the ultimate goal.

(P249) Investigation of the derivatization efficiencies of glycosaminoglycan disaccharides with different reducing end chemistries

Adam Hawkridge[1], Nidhi Naik[1,2]

[1]*Department of Pharmaceutics, Virginia Commonwealth University,* [2]*Pharmaceutical Engineering Program, Virginia Commonwealth University*

Glycosaminoglycans (GAGs) including heparin/heparan sulfate, chondroitin/dermatan sulfate, and keratan sulfate are linear polysaccharides that present unique glycoanalytical challenges compared with branched complex carbohydrates (e.g., N- and O-linked glycans). Common to both linear and branched complex carbohydrates is the aldehyde functional group at the reducing end following chemoenzymatic digestion or release, respectively. The aldehyde can be selectively derivatized with amine-, hydrazide-, and oxyamine-containing chemical tags which thereby facilitate enhanced UV-vis, fluorescence, and mass spectrometric analysis. Several tagging strategies have been developed for this purpose and each displays different analytical figures of merit yet their derivatization efficiencies with GAG disaccharides is not well-documented. We investigated the derivatization efficiencies of five reducing end reagents (aniline, 2-AA, BODIPY-hydrazide, benzhydrazide, and O-benzylhydroxylamine) with heparin ($n = 12$) and chondroitin sulfate ($n = 7$) disaccharides using protocols from the literature. Derivatization efficiencies were determined using a Vanquish UHPLC-UV-vis system fitted with a 2.1 x 150 mm GlycanPac AXH-1 HILIC column (40°C; 300 ul/min) and coupled to a Thermo Q Exactive tandem mass spectrometer. The derivatized-to-underivatized amounts of disaccharides were quantified using UV-vis (232 nm) while the mass spectrometer was used to identify each species. We will present the results from each set of experiments as well as highlight critical analytical figures of merit, observed side reactions, and potential improvements to existing protocols.

(P250) One-Step Selective Labeling of Native Cell-Surface Sialoglycans by Exogenous α2,8-Sialylation

Jonathan Babulic[1], Joshua Kofsky[2], Marie Boddington[1], Youjin Kim[1], Emmanuelle Leblanc[1], Sophie Emberley-Korkmaz[1], Che Colpitts[1], Chantelle Capicciotti[1,2,3]

[1]*Department of Biomedical and Molecular Sciences, Queen's University, Kingston, K7L 3N6, Canada,* [2]*Department of Chemistry, Queen's University, Kingston, K7L 3N6, Canada,* [3]*Department of Surgery, Queen's University, Kingston, K7L 3N6, Canada,*

Exo-enzymatic glycan labeling strategies have emerged as versatile tools for efficient and selective installation of glycan terminal motifs onto live cell-surfaces. Through employing specific enzymes and nucleotide sugar probes, cells can be equipped with defined glyco-epitopes for modulating cell function or selective visualization and enrichment of glycoconjugates. In this presentation, we report the *Campylobacter jejuni* sialyltransferase Cst-II I53S as a tool for cell-surface glycan modification to install the biologically relevant α2,8-disialyl motif on to sialylated glycans. Labeling with Cst-II was achieved with biotin- and azide-tagged CMP-Neu5Ac derivatives on a model glycoprotein and on native sialylated cell-surface glycans across a panel of cell lines. By examining the specificity of Cst-II on cell surfaces, it was revealed that the α2,8-sialyltransferase primarily

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labeled N-glycans, with O-glycans labeled to a lesser extent, and there was an apparent preference for α2,3-linked sialosides. Additionally, this presentation will discuss an interesting mechanistic intermediate that was discovered for Cst-II, along with ongoing follow-up studies for its characterization. This cell-surface glycan editing approach with Cst-II thus broadens the scope of tools for selective exo-enzymatic labeling of native sialylated glycans and expands the exo-enzymatic labeling toolkit to include installation of α2,8-disialyl epitopes.

(P251) Developing a cell-free platform to identify high efficiency glycosylation sites in glycoconjugate vaccine carrier proteins

Zachary Shaver[1,2,3], Derek A. Wong[1,2,3], Katherine F. Warfel[1,2,3], Michael C. Jewett[1,2,3]

[1]*Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA,* [2]*Center for Synthetic Biology, Northwestern University, Evanston, IL, USA,* [3]*Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA,*

Glycoconjugate vaccines, consisting of O-antigen glycans or capsular polysaccharides from pathogenic bacteria conjugated to immunogenic carrier proteins, are effective tools to prevent bacterial infections. Conjugate vaccines are commonly synthesized by chemical conjugation, through which pathogen glycans are attached non-specifically to a carrier protein. However, this method can result in the loss of protective immune epitopes. Enzymatic conjugation methods have instead been developed to site-specifically conjugate glycan to carrier protein using an oligosaccharyltransferase, such as PglB from *Campylobacter jejuni*. This enzymatic method was first performed *in vivo* in *Escherichia coli* but has recently been adapted to *in vitro* cell-free systems, allowing for a higher-throughput screening of vaccine design variables. One such variable is the precise location of glycosylation within a carrier protein. Modifying glycosylation location may reveal improvements to both glycosylation efficiency and glycoprotein immunogenicity. Here, we use enzymatic conjugation in cell-free systems to evaluate efficiency of glycosylation of 41 conjugation sites throughout the carrier protein *Haemophilus influenzae* protein D. Glycosylation sites were designed by placing the sequon "DQNAT," recognized by PglB, at each desired location. This screen was carried out using AlphaLISA, a bead-based luminescence assay, to detect carrier glycosylation. In total, glycosylation of 15 sites resulted in ≥5x signal above background, and glycosylation at each site was confirmed by western blot. CRM197 is another highly immunogenic carrier protein that is more commonly used in clinically licensed glycoconjugate vaccines. We additionally optimized reaction conditions to both express full-length CRM197 in cell-free reactions and glycosylate the full-length product with a pathogen glycan. This work reveals numerous precise glycosylation sites throughout a carrier protein with varying glycosylation efficiencies. Future work will adapt a similar screen to CRM197 for evaluation of glycosylation ability, efficiency, and immunogenicity.

(P252) *N*-linked glycosylation of structured protein domains in cell-free reaction environments

Erik Bidstrup[1], Kyle Hill[2], Alina Chisti[1], Weston Kightlinger[2], Matthew DeLisa[1,3,4]

[1]*Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, 120 Olin Hall, Ithaca, NY 14853, USA,* [2]*National Resilience Inc., 2100 Livingston St, Oakland, CA 94606, USA,* [3]*Nancy E. and Peter C. Meinig School of Biomedical Engineering, Cornell University, Weill Hall, Ithaca, NY 14853, USA,* [4]*Cornell Institute of Biotechnology, Cornell University, Ithaca, NY 14853,*

Synthetic glycobiology has the potential to revolutionize the design and production of glycoprotein therapeutics by controlling both the pattern and the location of their glycosylation,. One key tool of synthetic glycobiology, cell-free glycoengineering, has allowed for the precision remodeling of *N*-glycans to generate bespoke glycoproteins with desired properties such as improved stability and biological activity. Despite notable successes, installation of custom preformed *N*-glycans onto acceptor proteins in cell-free reaction environments remains an understudied and challenging endeavor. A core driver of this challenge is the dependence on membrane-embedded oligosaccharyltransferases (OSTs) whose activity is constrained by the accessibility of the acceptor protein modification site. Indeed, the bacterial OST PglB from *Campylobacter jejuni*, which is typically used in cell-free glycosylation systems, favors substrate proteins with extended consensus sequences ((D/E)X_1NX_2(S/T), $X_{1,2} \neq$ proline) in flexible, solvent-exposed motifs. Such requirements are not necessarily met by the minimal NXT consensus sequences found in many native glycoproteins of therapeutic importance (e.g., immunoglobulin G (IgG); RNase A) whose structured sequences are typically glycosylated via a co-translational mechanism. The PglB-mediated glycosylation of IgG and RNase A in glyco-competent *Escherichia coli* cells therefore suggests that partial or complete unfolding is a key feature of structured site glycosylation. It remains an open question whether co-translational glycosylation could be activated in cell-free glycosylation systems.

To deepen our understanding of this phenomenon, we took advantage of one-pot cell-free glycoprotein synthesis (CFGpS), which combines transcription, translation, and glycosylation in an open reaction environment. This was accomplished using *E. coli*-derived cell-free extracts enriched with glycosylation components such as PglB and lipid-linked oligosaccharides (LLOs)

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bearing preformed *N*-glycans for tight control of glycoprotein synthesis, glycan identity, and glycan transfer in a portable platform. By doing so, we demonstrated that the native structured glycosylation site in RNase A was efficiently *N*-glycosylated using CFGpS, but not when using an *in vitro* reconstituted system with purified, already folded RNase A. Subsequent investigation revealed that efficient glycosylation of structured sites in acceptor proteins produced in CFGpS was enabled by a co-translational, but not co-translocational, mechanism. The CFGpS platform was also found to promote similar co-translational glycosylation of the highly conserved structured glycosylation site at N297 in the Fc region of human IgG. Collectively, these results increase our understanding of co-translational glycosylation in cell-free reaction environments and mark an important step towards total cell-free biosynthesis of IgGs and other complex glycoprotein targets with controllable glycosylation.

(P253) Immune organoid-enabled glycoengineering of next-generation conjugate vaccines

Sophia Hulbert[1], Matthew P. DeLisa[2]

[1]Biochemistry, Molecular, and Cell Biology, Cornell University, Ithaca, NY, USA, [2]Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, USA

Most pure carbohydrates are weakly immunogenic and do not elicit B cell memory in high-risk groups, thereby limiting their use as vaccine candidates. A common strategy for enhancing the immunogenicity of carbohydrates and evoking carbohydrate-specific immunological memory is to covalently attach the carbohydrate to a carrier protein, which provides T cell epitopes for a typically T cell-independent antigen. While such conjugate vaccines are a highly effective strategy for protecting against diverse pathogens, their production features key drawbacks. Conventional methods necessitate cultivating and processing hazardous pathogens, followed by labor-intensive isolation and random chemical conjugation of desired polysaccharides. The resulting glyco-products are highly heterogeneous in terms of glycan conjugation sites and linked polysaccharide length, rendering them difficult to characterize and making it challenging to precisely engineer the immune response. As such, the design of conjugates is often empirical, relying on laborious trial-and-error experimentation, and delaying the rollout of much-needed life-saving vaccines.

To develop next-generation conjugate vaccines, we sought to identify optimal conjugation sites in the carrier protein *Haemophilus influenzae* protein D (PD) that elicit superior immune responses. Specifically, we used shotgun scanning glycomutagenesis (SSGM) to generate a PD glyco-site library in which each member of the library carried a glycan acceptor site at a different amino acid location, collectively spanning all possible positions along the protein backbone. To enable biosynthesis and glycosylation of each library member, we employed glycoengineered bacteria to site-specifically outfit each PD library member with the O-antigen polysaccharide from *Francisella tularensis* (*Ft*O-PS). PD glyco-site variants that were compatible with *Ft*O-PS glycosylation were isolated by high-throughput screening using glycoSNAP (glycosylation of secreted *N*-linked acceptor proteins). To effectively test the resulting panel of vaccine candidates, we employed a three-dimensional biomaterials-based B-cell follicular organoid system that is able to predict conjugate immunogenicity through expression of hallmark immune markers in a manner that is known to correlate with *in vivo* humoral responses in mice. Using this system, we characterized the immunogenicity of conjugates as a function of *Ft*O-PS attachment site, with promising candidates undergoing validation using *in vivo* mouse models. Overall, this workflow should enable the generation and identification of next-generation conjugate vaccine candidates whose performance is optimized due to the location of glycan conjugation.

(P254) Expanding the Selective Cell-Surface Glyco-Engineering Toolbox to Interrogate Glycan-Mediated Interactions

Jonathan L. Babulic[2], Fabiola De Leon Gonzalez[1], Joshua M. Kofsky[1], Marie E. Boddington[2], Youjin Kim[2], Chantelle J. Capicciotti[1,2,3]

[1]Department of Chemistry, Queen's University, Kingston, ON K7L 3N6, Canada, [2]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON K7L 3N6, Canada, [3]Department of Surgery, Queen's University, Kingston, ON K7L 3N6, Canada,

The interactions of cell-surface glycans with proteins is central to a diverse range of physiological and pathological processes including cell-cell communication, molecular recognition, immunological responses, infectious diseases and cancers. However, identifying the precise endogenous glycan structures involved in these interactions, and the mechanisms by which they elicit function, is challenging. Novel chemical biology tools are needed to advance our understanding of glycans and glycan-protein interactions and meet the demand for new information of these important biomolecules. One strategy the Capicciotti Lab has been working towards is expanding the cell-surface glyco-engineering toolbox. We use chemo-enzymatic synthesis to prepare carbohydrate-based probes, which are then installed on cells using an enzymatic-based cell-surface glyco-engineering strategy. By harnessing the specificity of the glycosyltransferase enzymes used to install the probes, cell-surface glycans can be selectively

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edited in a motif-specific. Studies using cell-surface glyco-engineering to selectively install various sialic acid-based and LacNAc-based glyco-motifs with chemical reporters to visualize and characterize glycans and glycoproteins will be discussed. Additionally, the use selective exo-enzymatic glycan editing to install photo-crosslinkable probes to interrogate and capture glycan-protein interactions on cells will also be described, including the application of diazirine modified CMP-sialic acids to capture Siglec-mediated interactions. These techniques will enable the identification of functional glycan ligands of glycan-binding proteins, such as those involved in cancer immune evasion mechanisms and virus-host cell interactions, and they will facilitate the development of novel targets for therapeutic intervention.

(P255) GlyTouCan update version 3.2: new functionality and plans for major release 4.0

Akihiro Fujita[1,2], Thomas Masding[3], Masaaki Shiota[2], Masaaki Matsubara[4], Issaku Yamada[4], Kiyoko F. Aoki-Kinoshita[1,2,3]

[1]Faculty of Science and Engineering, Soka University, Tokyo, Japan, [2]Glycan & Life System Integration Center(GaLSIC), Soka University, Tokyo, Japan, [3]Graduate School of Science and Engineering, Soka University, Tokyo, Japan, [4]The Noguchi Institute, Tokyo, Japan,

We have been developing the GlyTouCan repository, whose mission is to assign and reference accession numbers to glycan structures. These accession numbers in GlyTouCan are linked not only to major glycan structure databases but also to databases in related fields, such as PubChem. The development of the WURCS format is a major factor in the technical aspects that support these efforts. However, some problems have arisen as a side effect because WURCS expresses detailed glycan structures from a chemical perspective.1) There are glycan structures that differ only in the anomer information at the reducing end, and the desired knowledge is separated and linked to each structure.2) Variations in the expression of glycan structures with monosaccharide compositions occur because it is a conceptual structure that includes actually existing structures. This variation arises due to differences in the researcher's viewpoints on monosaccharide conformation. The representation of these glycan structures changes because researchers understand glycan structures differently. Oftentimes "similar" glycan structures are registered, so we have been attempting to organize such structures to better access the most relevant entries.We have been implementing the concept of "archetype" glycans which represent groups of "similar" glycan structures, in an attempt to organize the structures in GlyTouCan and make it easier for users to access the knowledge they need. The latest update to GlyTouCan version 3.2 includes the experimental introduction of the "archetype" and various improvements to the previous system. In the future version 4.0, we plan to update the internal system to support the latest updates of the WURCSFramework and to smoothly perform updates such as the normalization of glycan structures.

(P256) Enzymatic transfer of a single GlcNAc residue to asparagine in a single-pot in vitro glycosylation reaction

Madison DeWinter[1,2], Derek Wong[1], Weston Kightlinger[3], Matthew DeLisa[4], Michael Jewett[1,5]

[1]Department of Chemical and Biological Engineering, Northwestern University, Chicago, IL, [2]Medical Scientist Training Program, Northwestern University, Chicago, IL, [3]National Resilience, Inc, La Jolla, CA, [4]Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithica, NY, [5]Department of Bioengineering, Stanford University, Palo Alto, CA,

Glycosylation is an important and complex post-translational modification present in many therapeutic proteins. Glycans perform many roles and are important in cell signaling, protein stability, protein function, and immunogenicity. Although glycosylation can be performed by eukaryotic cells during therapeutic protein production, this process relies on many competing glycosyltransferases, glycosidases, and other enzymes in a complex cellular environment thus creating a heterogenous distribution of glycans. *In vitro* glycosylation (IVG) presents an opportunity for more control of glycan composition, which could lead to better elucidation of specific glycan function and more homogenous biologic drug products. There is an interest in synthesizing eukaryotic N-linked glycans bottom up, but this relies on installing an N-acetylglucosamine (GlcNAc) at the reducing end for further elaboration into complex and consistent eukaryotic glycans. Current *in vitro* technology to install this monomer on asparagine amino acids is limited to multi-step enzymatic glycosylation and chemical modification or en bloc transfer of large glycans and subsequent glycosidase treatment. Here, we show *in vitro* transfer of a single GlcNAc residue to asparagine with a PglB-based oligosaccharyltransferase in a single-pot reaction. The enzyme is produced in a nanodisc-supplemented cell-free protein synthesis reaction, and subsequently used in an IVG reaction to transfer GlcNAc to an sfGFP acceptor protein with a C-terminal DQNAT sequon. Evidence of this transfer is given by mass spectrometry, and further characterization of mechanism of transfer, the optimization of *in vitro* glycosylation conditions, and screening of enzyme variants is presented in this work.

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(P257) Hyperactive GlcNAc-1-Phosphotransferase (S1S3 PTase) Dramatically Alters Glycosylation of Lysosomal Enzymes Leading to Enhanced Phosphorylation for Improved CI-MPR Binding and Cellular Uptake

Kylie Gray[1], Riley Marcinczyk[1], Jonathan Roberts[1], Uday Wanninayake[1], Vaughn Weaver[1], Michael DiGruccio[1], Shou Liu[1], Nastry Brignol[2], Osman Sheikh[2], Steven Ortemier[3], Clarissa Booth[3], Kathrine White[3], Russell Gotschall[1]

[1]*M6P Therapeutics Inc, St. Louis, MO, USA,* [2]*Amicus Therapeutics, Philadelphia, PA, USA,* [3]*Sanford Research, Sioux Falls, SD, USA,*

Soluble lysosomal enzymes are synthesized in the endoplasmic reticulum and transported to the Golgi network where their N-linked glycans are modified by resident carbohydrate processing enzymes. N-acetylglucosamine-1-phosphotransferase (PTase) catalyzes the transfer of an N-acetylglucosamine (GlcNAc)-linked phosphate group onto certain mannose residues of N-glycans. Then N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (NAGPA, aka Uncovering Enzyme) removes the covering GlcNAc to reveal manose 6-phosphate (M6P). This phosphorylation process is inherently inefficient and yields newly synthesized lysosomal enzymes with no or low levels of M6P. Historically it has been difficult to improve this phosphorylation process within cells, and this inefficiency is further exacerbated when lysosomal proteins are over-expressed for either enzyme replacement (ERT) or gene (GTx) therapies. The development of a hyperactive PTase (S1S3 PTase) has enabled the efficient phosphorylation of lysosomal enzymes for the development of either ERT or GTx therapies for lysosomal storage diseases. The oligosaccharide composition, Cation Independent Mannose 6-Phosphate Receptor (CI-MPR) binding and cellular uptake of 4 recombinant lysosomal enzymes co-expressed with S1S3 PTase has been evaluated. Co-expression of lysosomal enzymes with S1S3 PTase overcomes inherently poor phosphorylation to produce lysosomal enzymes with improved glycan phosphorylation, CI-MPR binding and cellular uptake. Specifically, glucocerebrosidase (M011), acid α-glucosidase (M021), α-galactosidase (M051), and palmitoly-protein thioesterase (M081), exhibit a significantly enhanced amount of bis-phosphorylated high mannose oligosaccharides compared to lysosomal enzymes expressed without S1S3 co-expression. This is confirmed by site-specific glycosylation analysis which indicates that glycosylation patterns are dramatically altered when enzyme are expressed in the presence of S1S3 PTase. Functionally, affinity chromatography using immobilized CI-MPR, demonstrates that the co-expression of the 4 lysosomal enzymes with S1S3 PTase increased the amount of enzyme that can interact with CI-MPR. The higher percentage of molecules able to bind CI-MPR in combination with the higher levels of bis-phosphorylated glycans lead to significantly better affinity for the CI-MPR which results in more efficient cellular uptake. Limited *in vivo* data illustrating how the increased phosphorylation and improved CI-MPR interaction mediated by S1S3 translates to improved therapies in relevant animal models.

(P258) Synthetic Strategies for Glycans On Demand

Nicola Pohl[1]

[1]*Indiana University,*

The incredible diversity of glycans makes a general approach to the synthesis of these structures challenging. The paradigm used for automated peptide and nucleic acid synthesis—a few building blocks ready to string together on a machine—would require thousands of protected carbohydrate building blocks to implement. This talk demonstrates progress toward a new paradigm that relies on the development of automated procedures for the production of carbohydrate building blocks on demand. Automated continuous flow procedures allow easy scalability of these blocks. These methods can also produce glycosylated amino acids to drive new glycoproteomics analyses workflows.

(P259) Construction of structurally defined heparan sulfate and chondroitin sulfate microarray

Guowei Su[1], Maurice Horton[2], Yongmei Xu[1,2], Vijayakanth Pagadala[1], Truong Pham[1], Jian Liu[1,2]

[1]*Glycan Therapeutics, LLC,* [2]*UNC Eshelman School of Pharmacy, University of North Carolina*

Heparan sulfate (HS) and chondroitin sulfate (CS) are two types of heterogeneous, extracellular glycans that interacts with proteins and other molecules affecting many biological processes. The specific binding motifs of HS/CS protein interactions are of interest but have not been extensively characterized. Their structural complexity has limited an understanding of their biological roles and structure-function relationships. Recently, chemoenzymatic synthesis of HS and CS have been employed to produce specific HS/CS structures that can otherwise be difficult to produce. Glycan microarrays are valuable tools that can be used to probe the interactions between glycans and their ligands while relying on relatively small amounts of samples. We have developed a microarray with up to 96 structurally defined HS oligosaccharides with a size range from tetrasaccharides (4-mer)

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to octadecasaccharides (18-mer), representing many different sulfation types that can be found in nature. Fluorescently labeled antithrombin III (AT) and fibroblast growth factor-2 (FGF2) were screened against these 96 different HS structures under three different printing concentrations to confirm the utility of this microarray. Specific sulfation patterns were found to be important for binding to these proteins and results are consistent with previous specificity studies. Here, a microarray with structure defined CS oligosaccharides ranging from 5-mer to 19-mer in size, including CS backbone, CS-A, CS-C and CS-E is under developing. A group of CS binding proteins/antibodies were screened against the CS microarray to test the sensitivity. This high-throughput microarray technology can allow researchers to identify the specific saccharide sequences bound to the protein of interest. This technology is now available from Glycan Therapeutics, which will accelerate heparan sulfate-related research and the effort to develop and design heparan sulfate-based therapeutics.

(P260) Collaboration, Service and Trainings at the Complex Carbohydrate Research Center

Stephanie Archer-Hartmann[1], Christian Heiss[1], Artur Muszynski[1], Ian Black[1], Liyanage Devthilini Pasasum Fernando[1], Krishna Moorthy Manchuri[1], Jiri Vlach[1], Li Tan[1], Varughese Mulamoottil[1], Nathan Murray[1], Griffin Keiser[1], Bhoj Kumar[1], Xu Yang[1], Lei Huang[1], Jie Lun Cheng[1], Sergei Shalygin[1], Mehrnoush Taherzadeh Ghahfarrokhi[1], Ambrish Kumar[1], Tatiana Rojo Moro[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center, UGA, Athens, GA,*

For more than 30 years, the Complex Carbohydrate Research Center (CCRC) has collaborated with universities, federal agencies, and industry from the US and other countries on detailed structural characterization of glycoconjugates. We have expertise in a full range of analytical methodologies needed to conduct comprehensive research in glycobiology. We are equipped with the state-of-the-art instrumentation including a Thermo Ascend- MS, Thermo Orbitrap-Eclipse MS, Thermo Orbitrap-Fusion MS, Waters Synapt XS MS, Bruker RapiFlex MALDI-MS, AB SCIEX TOF/TOF 5800 MALDI-MS, SPR, multiple analytical and preparative separations options, and GC-MS. The CCRC's NMR facility offers outstanding resources for high-field NMR spectroscopy including 900 MHz, 800 MHz, three 600 MHz and a 500 MHz spectrometer. These spectrometers are equipped with variety of probes including a HRMAS probe for solid state analysis and several H-C/H-N cryoprobes and HCN cryoprobes dedicated to biomolecular NMR applications.

Collaborative projects with the CCRC can be very diverse and can include: Glycomics and glycoproteomics, isolation and analysis of Glycosaminoglycans (GAGs) and GAG-derived products, polysaccharides and Lipopolysaccharides (LPS), peptidoglycans, and glycolipids. Staff at the CCRC-AST also excel in the development of novel characterization and isolation methods, depending on the individual client's needs.

The CCRC offers yearly training courses on **techniques for structural characterization glycans of glycoproteins, glycolipids, polysaccharides, and GAGs, as well as courses on mass spectrometry techniques and software analysis.** Training participants will perform hands-on experiments and analysis by mass spectrometry, GC/MS, HPLC-UV and fluorescent detection, HPAEC-PAD, lectin blotting, and thin layer chromatography. The hands-on experience is further enhanced by lectures from faculty on various fields of glycobiology.

(P261) Drastically improved recoveries in glycosyl composition and linkage analysis of insoluble and acidic polysaccharides

Ian M. Black[1], Ikenna E. Ndukwe[1,2], Jiri Vlach[1], Jason Backe[1], Breeanna R. Urbanowicz[1], Christian Heiss[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens GA, 30602, USA,* [2]*Current address: Pivotal Attribute Sciences, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320*

The structural characterization of polysaccharides requires accurate glycosyl composition and linkage analysis to useful for gaining understanding of the structural diversity and biological importance of polysaccharides. However, limited solubility of polysaccharides often leads to incomplete depolymerization and inaccurate results. This is especially problematic in the case of mixtures of polysaccharides with differing solubility, which is usually the case in biological samples, resulting in data that do not reflect the true composition of the sample. Similarly, the methylation analysis of acidic polysaccharides is hampered by their poor solubility in DMSO, so that uronic acids are vastly underrepresented in the linkage analysis results. In addition, β-elimination during permethylation presents a continuing challenge in the linkage analysis of acidic polysaccharides. We have now discovered that these challenges can be overcome by pretreating insoluble or acidic polysaccharides by acetylating them after dissolution in the ionic liquid 1-ethyl-3-methylimidazolium acetate [Emim][Ac]. This acetylation greatly increases solubility in methanol (for composition analysis) or DMSO (for linkage analysis) and leads to vastly improved recoveries regardless of difference in water-solubility of the initial polysaccharide constituents of the sample. The composition and linkage data obtained by gas chromatography-mass spectrometry after acetylation in ionic liquid thus lead to a much more accurate representation of the monosaccharide and linkage composition of the sample.

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(P262) Exploring Novel Glycosyltransferases from Mollusc-Origin: Unravelling Molluscan Glycosylation Abilities and their Biotechnological Potential

Marilica Zemkollari[1], Reingard Grabherr[2], Erika Staudacher[1]

[1]*Department of Chemistry, University of Natural Resources and Life Sciences, Vienna,* [2]*Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna*

Proteins are essential tools in biotechnology, enabling the production of therapeutics, vaccines, and diagnostic reagents. However, their utility is constrained by stability and solubility issues, potential immunogenicity, and altered biological activity. Over the years, various protein engineering methods have emerged to address these limitations, and one particularly promising approach is glycoengineering. Glycoengineering involves the manipulation and modification of the glycans attached to proteins or other molecules. Glycans play crucial roles in various biological processes, including cell signalling, immune response, and protein folding. By modifying these glycans, desired properties or functions can be achieved. Since glycosylation is not template-driven, presence of specific glycotransferases and glycosidases is needed.

On the other hand, Mollusca is one of the most diverse groups of animal kingdom and they display significant functions in many ecosystems as they are important filter feeders and decomposers, but they are also pests in agriculture and intermediate hosts of many human and cattle parasites. Their ability to produce complex glycans is a fundamental aspect of their biology. Glycans are involved in cell-cell communication, host-pathogen recognition and orchestration of immune response. In many cases molluscs display similar glycan structures with the human parasites they host. This makes them an interesting system to study glycosylation.

In this case, the identification of two novel core 1 β-1,3-galactosyltransferses (T-synthase) from *Pomacea canaliculata* and *Crassostrea gigas* and an α-1,2-fucosyltransferase from *Crassostrea gigas* presents exciting opportunities for biotechnological applications but also gives a better understanding of the glycosylation processes in molluscs and sheds light on their adaptation and survival abilities. Studying the diversity of glycosylation in different organisms can also provide valuable insights into the evolution of glycosylation pathways and their functional significance in different biological groups.

The sequences of the enzymes were identified by homology search. The B. glabrata T-synthase (QXN57605.1) and the rabbit alpha-1,2-fucosyltransferse (NP_001075872.1) were used as templates. The coding sequences were synthesised and expressed in Sf9 cells. The expression product of the putative T-synthases displayed core 1 β1,3-galactosyltransferase activity using pNP-α-GalNAc as the substrate and the putative α-1,2-fucosyltransferase was able to transfer fucose from UDP-fucose to lactose and complex N-glycan structure (Galβ1,4-GlcNAcβ1,2Manα1,6(Galβ1,4-GlcNAcβ1,2Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc-Asn). Biochemical characteristics and structural features where analysed and compared with previously characterised enzymes from other species.

In this study, we present the identification and characterization of novel T-synthases and the first ever mollusc-origin fucosyltransferase, providing insights into molluscan glycosylation abilities, expanding our knowledge of these enzymes across species, and offering new potential tools for biotechnological applications.

(P263) Glycan-dependent Affinity Purification Mass Spectrometry (GAP-MS) provides novel insights into glycoprotein interaction network

Yixuan Axe Xie[1,2], Xingyu Liu[1], Siyu Chen[2], Carlito B. Lebrilla[2,3], Benjamin A. Garcia[1]

[1]*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110, United States,* [2]*Department of Chemistry, University of California, Davis, California 95616, United States,* [3]*Department of Biochemistry, University of California, Davis, California 95616, United States,*

Protein-protein interactions define cellular processes and are the foundations for proteins to execute their functions. However, the cell membrane is composed of a layer of the glycocalyx, which forms a highly interactive network, and the role of glycan in such interactions is often overlooked due to the limitation of the methods. In this research, we report a novel workflow termed “glycan-dependent affinity purification mass spectrometry (GAP-MS)” to investigate the glycoprotein interactome on the cell membrane quantitively. We first employed a toolbox of glycan mediators to treat HCT116 cells to generate a variety of “glycan phenotypes”, including high-mannosylated, undecorated, fucosylated, sialylated, and sialofucosylated, on the cell membrane. The treatment condition was optimized based on the MS-based glycomic analysis. Subsequently, we investigated the glycan-dependent interactions of CD147 with an HaloTag as a proof of concept. We employed fluoresce imaging to evaluate the integration of the bait CD147 protein. As a result, the signals from bait protein were merged well into the cell membrane, which confirmed the expression and subcellular localization of CD147. The interacting partners of CD147 were pulled down by performing affinity purification, while we employed sequential window acquisition of all theoretical fragment-ion spectra (SWATH) scans for identifying and quantifying the enriched proteins. As a result, a comprehensive network of CD147 interactions with the added dimension of glycosylation was revealed using our method (e.g., protein interactions dependent on the glycosylation state of the protein). For example, two known interactors of CD147, epidermal growth factor receptor (EGFR) and CD44 were identified. In addition, their interactions were restricted with sialic acid-containing glycans and were able to be strengthened after treatment

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with kifunensine to generate high-mannose glycans. More importantly, by employing different bait glycoproteins, we illuminated a comprehensive panorama of cell surface glycoprotein networks. This approach offers novel perspectives into glycan-dependent interactions among cell surface protein networks.

(P264) Functions of sialidase revealed by sialidase activity imaging probe

Akira Minami[1], Yuuki Kurebayashi[1], Tadanobu Takahashi[1], Tadamune Otsubo[2], Kiyoshi Ikeda[2], Takashi Suzuki[1], Hideyuki Takeuchi[1]

[1]*Department of Biochemistry, Graduate School of Pharmaceutical Sciences, University of Shizuoka,* [2]*Department of Organic Chemistry, School of Pharmaceutical Sciences, Hiroshima International University*

Sialidase cleaves sialic acid residues from glycans such as glycoproteins and glycolipids. In the brain, sialidase is essential for hippocampal synaptic plasticity, learning and memory, and synaptic transmission. Some artificial substrate that can be used for imaging sialidase enzyme activity in mammalian tissues includes PNP-Neu5Ac, X-Neu5Ac, and a combination of X-Neu5Ac and fast red violet LB. However, these substrates have low sensitivity and specificity for staining mammalian sialidase activity. Since the mammalian sialidase activity is significantly weaker than that of viruses and bacteria, it was difficult to stain mammalian sialidase activity using classical substrates. Thus, we have developed a BTP3-Neu5Ac with high specificity and sensitivity for mammalian sialidase activity (PLOS ONE, 2014). Staining of rat hippocampus with BTP3-Neu5Ac revealed relatively intense sialidase activity at the nerve terminals of mossy fibers, the major excitatory nerve in the hippocampus. Sialidase activity at the mossy fiber terminals increases rapidly by neuronal depolarization (J. Biol Chem., 2017). Since sialidase inhibits glutamate release from mossy fiber terminals, the increase in sialidase activity coupled with neuronal activity is presumed to be part of a negative feedback function on the glutamate release (J. Biochem., 2018). Mammalian tissues other than the brain were also stained with BTP3-Neu5Ac. Staining of the mouse pancreas revealed intense sialidase activity in the pancreatic islets. Based on this staining information, we investigated the role of sialidase on insulin release. It was found that a sialidase inhibitor can be used as an anti-diabetic drug that can avoid hypoglycemia, a serious side effect of insulin secretagogues (Sci. Rep., 2020). Staining of the rat skin showed that a lower layer of the dermis and muscle showed intense sialidase activity. Cleavage of sialic acid from microfibrils by the sialidase isozyme Neu1 has previously been reported to promote the formation of elastic fibers. In the present study, we also showed that not only Neu1 but also Neu2 would be involved in elastic fiber assembly (Sci. Rep., 2021). BTP3-Neu5Ac is useful in understanding the function of sialidase (Int. J. Mol. Sci., 2021).

(P265) Dysregulated O-GlcNAcylation is a molecular link to Alzheimer's disease

Chia-Wei Huang[1,2], Rebecca Lee Mai[1,3], Gerald W. Hart[1,2]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA,* [2]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA,* [3]*Department of Biology, University of Georgia, Athens, GA, USA,*

The reduced O-GlcNAcylation has been correlated with sporadic Alzheimer's disease (sAD) pathogenesis, the most common neurodegenerative disease. Decreased O-GlcNAcylation in sAD likely arises from impaired glucose metabolism, which is preceded by regional hypoperfusion, and occurs years before onset of symptoms. However, the lack of a human model impedes the understanding of mechanisms underlying sAD and the roles of O-GlcNAc in AD pathology. Here, we present a human neuronal model in which the pathological features, including increased tau phosphorylation and amyloid beta production, are reproduced only by glucose deficiency, providing a better representation of sAD as a metabolic disease. We generated human cortical neurons from human pluripotent stem cells (hPSCs) and cultured mature neurons in glucose reduction media. We found a narrow window of glucose concentration that induces neurodegeneration along with AD-like hallmarks in hPSC-derived cortical neurons. In contrast, hPSC-derived cerebellar neurons, which are less susceptible to AD pathology, remained unaffected by glucose reduction in terms of AD-like changes. With this cell model, we further explored that reduced O-GlcNAcylation through, in part, mitochondrial dysfunction, is involved in early stages of the onset of sAD-like changes. Finally, we demonstrated the therapeutic potential of pharmacological inhibiting O-GlcNAcase in alleviating AD-like biochemical changes in our model. Considering the sex and regional differences in the incidence and progression of AD, we also compared the levels and patterns of O-GlcNAcylation in male versus female hPSC-cortical neurons and mouse brains. Generally, female cortical neurons and brains have higher O-GlcNAcylation and OGT levels than that in males. Elevated OGT level in females is probably due to post-transcriptional regulation. With two-dimensional electrophoresis, we further revealed that male and female brains have different O-GlcNAcylated protein patterns. Moreover, although O-GlcNAcylation levels in female brains are globally higher, regional differences are observed. The cortex, hippocampus, and cerebellum are enriched with a higher abundance of O-GlcNAcylated proteins in females, whereas males exhibit increased O-GlcNAcylation levels in the striatum. This might be associated with sex-difference in neurodegeneration. Taken together, we established a human model that mimics the main features of AD-like changes, which agrees with clinical observations of sAD patients. Our results also suggest that dysregulated O-GlcNAc might be a direct molecular link between hypometabolism and sAD-like alternations, and that O-GlcNAc could play a mechanistic

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role in sex and region-specific vulnerabilities to AD. Therefore, this platform can serve as a tool to better understand molecular processes involved in sAD and for drug development.

Supported by the Georgia Research Alliance and NIH R01DK61671. *Dr. Hart receives a share of royalty received on sales of the CTD 110.6 antibody, managed by JHU.*

(P266) Neurodevelopmental consequences of disrupting the UDP-galactose translocator in mice

Hyojung Yoon[1], Amanda Ringland[1], Rachel Corrigan[1], James Anderson[1], Cindy Huynh[1], Sahib Sran[1], Foued Amari[2], Min Chen[2], Vincenzo Coppola[2], Soad Elziny[3], Peter Crino[3], Tracy Bedrosian[1,4]

[1]*Institute for Genomic Medicine, Nationwide Children's Hospital, Columbus, OH,* [2]*Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH,* [3]*Department of Neurology, University of Maryland School of Medicine, Baltimore, MD,* [4]*Department of Pediatrics, The Ohio State University, Columbus, OH,*

SLC35A2 encodes a UDP-galactose transporter that aids in glycan synthesis by providing galactose to the Golgi apparatus and endoplasmic reticulum. Genetic variants resulting in SLC35A2 loss of function have been clearly associated with disease, but the pathogenesis remains understudied. SLC35A2-congenital disorder of glycosylation (SLC35A2-CDG, also known as congenital disorder of glycosylation type llm), is an X-linked disorder associated with infantile seizures, hypsarrhythmia, hypotonia, and brain malformation. Also, somatic *SLC35A2* variants were recently identified as a genetic marker for mild malformations of cortical development with oligodendroglial hyperplasia in epilepsy (MOGHE). Although MOGHE and CDGs are highly associated with early-onset epilepsy and neurological abnormalities, little is known about the role of SLC35A2 in cortical development or the contributions of abnormal neurons or oligodendrocytes to seizure activity. To study these questions, we generated a novel floxed *Slc35a2* mouse line and crossed it to the established *Emx1*-Cre and *Olig2*-Cre mouse lines to generate a forebrain knockout and oligodendrocyte knockout, respectively. Knockout of *Slc35a2* in the *Emx1* lineage (i.e., forebrain excitatory neurons, astrocytes, and oligodendrocytes) resulted in reduced lifespan, abnormal behavior, mild cortical malformation, and severe, progressive seizures. Specific knockout of *Slc35a2* in oligodendrocytes did not result in seizures, at least in preliminary experiments. These results demonstrate a direct causal role for SLC35A2 in developmental brain malformations and epilepsy and provide the first evidence that affected neurons may be required for the expression of seizures in MOGHE. The novel SLC35A2 conditional knockout mouse will be a valuable tool for future investigation into the pathophysiology of MOGHE and SLC35A2-CDG, as well as provide new insights into the importance of glycosylation in brain development.

(P267) Decoding Glycomics: Differential Expression Reimagined

Jon Lundstrøm[1,2], James Urban[1], Daniel Bojar[1]

[1]*Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden,* [2]*Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Gothenburg, Sweden*

Glycomics, the comprehensive study of all glycan structures in a sample, is a rapidly expanding field with substantial relevance for understanding physiology and disease mechanisms. However, the complexity of glycan structures and glycomics data interpretation present significant challenges, especially when it comes to differential expression analysis. Here, we present a novel computational framework for differential glycomics expression analysis. Our methodology encompasses specialized and domain-informed methods for data normalization and imputation, glycan motif extraction and quantification, differential expression analysis, motif enrichment analysis, time series analysis, and meta-analytic capabilities, allowing for synthesizing results across multiple studies. All methods are integrated into our open-source glycowork package, facilitating performant workflows and user-friendly access. We demonstrate these methods using dedicated simulations and various glycomics datasets. Our rigorous approach allows for more robust, reliable, and comprehensive differential expression analyses in glycomics, contributing to the advancement of glycomics research and its translation to clinical and diagnostic applications.

(P268) The remarkably resilient blood-brain barrier glycocalyx

Reid Larsen[1], Jeffrey Esko[2], Richard Daneman[3,4]

[1]*Biomedical Sciences Graduate Program, University of California, San Diego,* [2]*Department of Cellular and Molecular Medicine, University of California, San Diego,* [3]*Department of Pharmacology, University of California, San Diego,* [4]*Department of Neurosciences, University of California, San Diego,*

The blood-brain barrier (BBB) glycocalyx is the sugar rich structure lining the luminal surface of cerebral blood vessels that acts as the first barrier in the BBB. The BBB describes the combination of properties that tightly regulates blood vessel permeability and is exclusively found in the central nervous system vasculature. Despite its importance in the BBB, the glycocalyx is poorly

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understood; we do not fully understand its molecular composition or how it differs from the glycocalyx in other organs. However, its degradation has been shown to increase BBB permeability and neuroinflammation.

To better understand the structure of the BBB glycocalyx, we performed electron microscopy on the glycocalyx in brain, heart, and muscle tissues from healthy mice. We found that the BBB glycocalyx in the brain was thicker than the glycocalyx in the heart and muscle. Interestingly, contrary to the peripheral vasculature in inflammation, the structure of the BBB glycocalyx structure remains largely unchanged in several severe neuroinflammatory models. Together these findings illustrate yet another unique property of the BBB and suggest that the BBB glycocalyx may contribute to the tight regulation of molecules and cells between the blood and the brain. Additionally, preliminary RNA sequencing and glycomics investigations have shown that the BBB glycocalyx may be enriched in heparan sulfate and sialic acid. In future studies, we will investigate the role of these glycans in the BBB glycocalyx, the barrier contribution of the glycocalyx to the BBB, and how its molecular composition and function change in neuroinflammatory disease.

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(P269) Allosteric Regulation of Ligand Binding to the Cation-independent Mannose 6-Phosphate Receptor

Richard Bohnsack[1], Sandeep Misra[2], Jianfang Liu[3], Mayumi Ishihara-Aoki[1], Michaela Pereckas[1], Kazuhiro Aoki[1], Gang Ren[3], Joshua Sharp[2], Nancy Dahms[1]

[1]*Medical College of Wisconsin, Milwaukee, WI,* [2]*University of Mississippi, Oxford, MS,* [3]*The Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley, CA,*

The cation-independent mannose 6-phosphate receptor (CI-MPR) plays a critical role in the biogenesis of lysosomes by transporting newly synthesized mannose 6-phosphate (M6P)-containing lysosomal enzymes (LEs) to endosomal compartments. CI-MPR is used clinically for patients with lysosomal storage diseases due to its ability to internalize recombinant LEs administered intravenously during enzyme replacement therapy. CI-MPR binds several extracellular ligands in a glycan-independent manner. One such ligand is the peptide hormone insulin-like growth factor 2 (IGF2), where CI-MPR modulates embryonic growth and fetal size by downregulating circulating levels of IGF2. CI-MPR's extracellular region is comprised of 15 homologous domains with binding sites for M6P-containing ligands located in domains 3, 5, 9, and 15, while the IGF2 binding site maps to domain 11. Our previous studies of a construct encoding human CI-MPR's domains 1–5 support that its five N-terminal domains undergo significant conformational changes due to pH and ligand binding (PMID: 32908216). Although CI-MPR contains binding sites for different ligands, how a particular ligand affects the receptor's conformation or its ability to bind other ligands is poorly understood. To address these questions, we used affinity-purified soluble CI-MPR (sCI-MPR) from newborn calf serum. The primary sequence and N-linked glycosylation of the purified receptor were assessed by mass spectrometry. Detected peptides from tryptic digests encompass nearly the entire extracellular region of the mature protein. We determined the glycosylation status of sCI-MPR's 19 potential N-linked glycosylation sites. Most sites are either fully or mostly glycosylated with complex structures. Five of the identified sites carry high-mannose type or hybrid-type glycans as major glycan components. Using surface plasmon resonance, we show that IGF2, but not the closely related IGF1, allosterically inhibits the ability of sCI-MPR to bind two different LEs. Two approaches were taken to probe conformational changes in sCI-MPR. First, hydroxyl radical protein foot printing experiments were carried out with sCI-MPR in the presence and absence of IGF2. Changes in side-chain oxidation were detected in peptides near the IGF2-binding site in domain 11, as expected, and in the distal N-terminal region of the receptor. In the second approach, negative-stain electron microscopy studies comparing the size of the sCI-MPR in the presence and absence of IGF2 show that the IGF2-bound structure has a more compact configuration. Our analysis of the cryogenic electron tomography 3D reconstructions of domains 4–14 of human CI-MPR bound to IGF2 (PDB 6UM2) shows that the M6P binding site in domain 9 is occluded in the IGF2-bound conformation. Together, our results support the hypothesis that the ability of IGF2 to inhibit LE binding is due to global conformational changes of CI-MPR induced by IGF2 binding. (NIH R01DK042667 and 1S10OD028640 to ND)

(P270) Storage stability and HILIC-UHPLC-FLR analysis of immunoglobulin G N-glycome from saliva

Ivan Gudelj[1,2], Barbara Radovani[1], Gordan Lauc[2,3]

[1]*Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, Rijeka, Croatia,* [2]*Genos Glycoscience Research Laboratory, Borongajska cesta 83h, Zagreb, Croatia,* [3]*Faculty of Pharmacy and Biochemistry, University of Zagreb, Ante Kovačića 1, Zagreb, Croatia,*

Immunoglobulin G (IgG) is the most abundant antibody in the blood and plays a critical role in host immune defense against infectious agents. Glycosylation is known to modulate the effector functions of IgG and is involved in disease development and progression. It is no surprise that the N-glycome of IgG from plasma has already been proposed as a biomarker for various physiological and pathological conditions. However, because saliva is easy to collect, it could be useful for exploring

the functional role of salivary IgG N-glycosylation and its potential as a diagnostic biomarker. Therefore, in this study, we described a method for N-glycome analysis of IgG from saliva samples. The method we developed involved bead-based immunoprecipitation of IgG from saliva followed by enzymatic deglycosylation and the glycomic analysis of labeled N-glycans by ultrahigh performance liquid chromatography based on hydrophilic interactions with fluorescence detection (HILIC-UHPLC-FLR). Using the developed method, we compared IgG N-glycan profiles from saliva with those from plasma and found that salivary IgG exhibited a lower degree of monogalactosylation and sialylation, suggesting that saliva may not be a direct substitute for plasma in this context. In addition, we assessed the stability of salivary IgG N-glycan profiles under different storage conditions and evaluated the effects of using a saliva preservation medium. This study provides an ultrasensitive UHPLC method for the analysis of total IgG N-glycosylation from saliva, gives insight into storage stability of salivary IgG, and highlights its (dis)advantages for further biomarker-related research.

(P271) A sulfoglycosidase from *Bifidobacterium bifidum* is involved in the degradation of sulfated mucin *O*-glycans in the intestine

Toshihiko Katoh[1], Chihaya Yamada[2], Toma Kashima[2], Mikiyasu Sakanaka[1], Ikuo Kimura[1], Keith A. Stubbs[3], Shinya Fushinobu[2], Takane Katayama[1]

[1]*Kyoto University,* [2]*The University of Tokyo,* [3]*The University of Western Australia,*

Mucin glycoproteins, components of mucus layers secreted from mammalian intestinal cells, undergo extensive modification of *O*-linked glycans. Recent studies have suggested that mucins serve as a nutrient for gut microbes, yielding beneficial metabolites like short-chain fatty acids, pivotal for host immune balance and energy. Therefore, understanding how gut microbes utilize mucin *O*-glycans enhances comprehension of the symbiotic relationship between humans and the microbiota and its health implications.

Bifidobacterium bifidum, a human commensal probiotic, possesses numerous cell-surface-anchored glycoside hydrolases (GHs) targeting mucin, which directly release monosaccharides and disaccharides from mucin extracellularly. GH20 BbhII, once thought to be a β-*N*-acetylglucosaminidase, was redefined as a sulfoglycosidase due to its higher efficiency toward 6-sulfated GlcNAc (GlcNAc-6S). *O*-Glycomic analysis of mucin glycoproteins following BbhII treatment revealed its specific action on GlcNAc-6S linkages attached at the non-reducing ends of glycans, such as sulfated core 2 structures. *N*-Acetylglucosamine 6-sulfate analogs, PUGNAc-6S and NAGT-6S, competitively inhibited BbhII. NAGT-6S also inhibited the degradation of the sulfated core 2 glycans by *B. bifidum*. The same glycan structure persisted in mice colonized with the *bbhII* mutant, but not the wild-type strain. In the conventional mice administered with the wild-type *B. bifidum*, the amount of free GlcNAc-6S increased, and an expansion of the genus *Bacteroides* was detected in the fecal microbiota. S1_11 sulfatase targeting GlcNAc-6S is conserved in various *Bacteroides* species, suggesting that GlcNAc-6S released by *B. bifidum* was metabolized by *Bacteroides* spp. This relationship was also observed in human adult fecal suspension and metagenome. These findings show a sulfoglycosidase-mediated alternative pathway for sulfated mucin *O*-glycan degradation and suggest the potential impact of sulfoglycosidase-mediated cross-feeding between microbes in the gut ecosystem.

(P272) Tackling a tripartite glycan conundrum: Flexibility/Sparse structural data/Signal resolution

Marcos Battistel[1], Mihajlo Novakovic[2], Hugo Azurmendi[1], Lucio Frydman[2], Darón Freedberg[1]

[1]*Laboratory of Bacterial Polysaccharides, Food and Drug Administration (FDA), Silver Spring, MD, 20993, United States,* [2]*Department of Chemical and Biological Physics, Weizmann Institute of Science, 7610001, Rehovot, Israel*

NMR structure-function studies of glycans face common, and often interconnected, challenges, namely: 1) discrimination of distinct conformations amongst a conformation-rich landscape, 2) limited structural data for more reliable molecular modeling and 3) spectral overlap, especially observed for homopolymers. We have attempted to address these issues by improving sensitivity and spectral resolution, though both cannot be simultaneously achieved. We show that labile 1H signals can help alleviate two of these challenges, increase the repertoire of structural data available and provide a path to discriminate potentially "biologically active" conformations, thus, improving the quality of both, the acquired data and the derived structural models. We also present our ongoing efforts to enhance the spectral resolution to enable the structural studies of larger homo- and hetero-oligosaccharides.

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(P273) Identifying urine N-glycans as Potential Biomarkers of Lupus Nephritis

Aaron O. Angerstein[1], Sandra Mungaray Sanchez[2], Danielle Beaufort[2], Tamara Knowling[2], Richard Drake[1]

[1]*Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA,* [2]*Department of Medicine, Rheumatology Division, Medical University of South Carolina, 68 President Street, Charleston, SC, 29425, USA*

Lupus Nephritis (LN) is a type of autoimmune kidney disease in the context of Systemic Lupus Erythematosus (SLE), impacting roughly 50% of SLE patients. SLE is a multi-system autoimmune disease predominantly affecting women of childbearing age. Diagnosis relies on clinical observations supported by lab studies and regular screenings such as urinalyses and glomerular filtration rate estimation, which are integral components of ongoing SLE and LN patient management. Given the complexity of LN and renal biopsy being the gold standard for diagnostics and prognostication, a less invasive urine-based diagnostic approach holds immense promise. The current study sought to address this by comparing N-glycans derived from glycoproteins in urine samples from individuals with LN, healthy controls (HC), and those with SLE without LN. The underlying hypothesis was that distinct N-glycan profiles associated with LN can be detected in urine, thus providing potential diagnostic biomarkers. Here, we analyzed 120 de-identified urine samples from the MUSC Biorepository: 40 LN, 40 HC and 40 SLE samples. Utilizing established urine processing methods, urine was concentrated and spotted in duplicate onto amine reactive slides for PNGase digestion to release N-glycans. Detection was performed by MALDI-QTOF mass spectroscopy and data analyzed using SCILS software.Our findings indicate that N-glycan profiles demonstrated the sensitivity required to differentiate LN from HC and SLE samples using one-way ANOVA analysis. A total of 87 unique N-glycans were identified, and of those, 30 were statistically significant using a p-value of 0.00005. In LN urine samples, sialylated and fucosylated triantennary N-glycans were particularly elevated. Furthermore, N-glycans associated with immunoglobulin G (IgG) displayed significant elevation in LN samples when compared to both HC and SLE samples.This study underscores the importance of urine glycomic profiles as a promising avenue for refining diagnostic procedures and uncovering novel biomarkers specific to diseases like Lupus Nephritis.

(P274) Glycanbuilder4Web: Development of a Web application for drawing and database searching of glycan structures

Naoki Kimura[1], Shinichiro Tsuchiya[1], Kiyoko F. Aoki-Kinoshita[2], Issaku Yamada[1]

[1]*The Noguchi Institute,* [2]*GaLSIC, Soka University*

Because of the complexity of the glycan structure depiction, we are continuing our efforts to enhance GlycanBuilder by making it compatible with the glycan notations WURCS and SNFG symbolic notation. Nevertheless, GlycanBuilder is currently limited to desktop use, and the setup of an environment in a compatible operating system is mandatory. To overcome this, we have developed GlycanBuilder4Web, a web-based version that does not require local installation. This online version makes glycan templates available as before, allows the addition and deletion of monosaccharides, and supports the incorporation of modifications. Notably, an improved user interface streamlines the process of editing glycan structures. In addition to these core GlycanBuilder features, seamless access to the GlyTouCan and GlyCosmos portal databases is also incorporated.

(P275) Semisynthetic Study of Interleukin-21(IL-21) using Novel Peptide Preparation Method

Nana Izawa[1], Yuta Maki[1,2], Ryo Okamoto[1,2], Yasuhiro Kajihara[1,2]

[1]*Department of Chemistry, Graduate School of Science, Osaka University,* [2]*Forefront Research Center, Graduate School of Science, Osaka University*

Interleukin-21 (IL-21) is a glycoprotein consisting of 138 amino acids and having an asparagine (*N*)-linked glycan. Also, IL-21 is involved in the regulation of the immune system. However, how the glycans affects this immune response has not been extensively investigated. In this study, we have investigated the semisynthesis of glycosylated IL-21 to elucidate the function of glycan. For the synthesis of glycosylated IL-21, peptide fragments at both ends of the *N*-linked glycan were prepared by a novel peptide preparation method combining *E. coli* expression and chemical synthesis. The resultant peptide was designed to install an intentional cystein and then was folded to make a native disulfide bond pattern. The successful formation of a native disulfide pattern gave a free cysteine which was subsequently examined chemical activation and thioesterification. In order to synthesize glycosyl whole polypeptide of IL21, we are currently investigating the introduction of a N-linked glycan using the chemistry of β-mercapto amino acids and long peptide-thioester obtained. In this presentation, we would like to discuss this new chemical approach in detail.

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(P276) Phyloglycomics: Understanding Vertebrate evolution from a glycome perspective

Abarna V.M. Murugan[1], Catherine Hayes[2], Frédérique Lisacek[2], Kiyoko F. Aoki-Kinoshita[3], Niclas G. Karlsson[4], Kathirvel Alagesan[1,13], Rebecca E. Griffiths[5], Tiago Oliveira[1,12], Pauline Dizon[1], Vienna Anderson[1], Kimberly A. Finlayson[6], Eloise Skinner[7,8], Daniel Bojar[9], Martin Linster[10], Jason P. van de Merwe[6], Gavin J.D. Smith[10], Samantha Richardson[11], Daniel Kolarich[1]

[1]Institute for Glycomics, Griffith University, QLD, Australia, [2]SIB Swiss Institute of Bioinformatics, Geneva, Switzerland, [3]Glycan & Life Systems Integration Center (GaLSIC), Soka University, Tokyo, Japan, [4]Faculty of Health Sciences, Department of Life Sciences and Health, Pharmacy, Oslo Metropolitan University, Oslo, Norway, [5]Australian Red Cross Lifeblood, Brisbane, QLD, Australia, [6]Australian Rivers Institute, School of Environment and Science, Griffith University, Gold Coast, Australia, [7]Department of Biology, Stanford University, Stanford, CA, 94305, USA, [8]School of Environment, Griffith University, Nathan, QLD, Australia, [9]Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden, [10]Emerging Infectious Diseases Program, Duke-NUS Medical School, Singapore, [11]School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC, Australia, [12]Present address: IMBA Institute of Molecular Biotechnology, Vienna, Austria, [13]Present address: Emmanuelle Charpentier's Laboratory Chariteplatz 1, Campus Charite Mitte 10117, Berlin, Germany,

Phylogenetic studies provide valuable insights into the glycome evolution from a genetic perspective. While genetic data has now become widely available from many species, systematic data on species-specific glycome traits remain sparse. Given that the glycosylation machinery is also regulated by factors beyond transcriptional control and is highly organ/body-fluid specific, it is unfeasible to accurately predict species-specific qualitative and quantiative glycome traits from genetic data alone. This lack of knowledge also impedes our understanding of glycome-relationships between species, and how inter-species glyco-trait similarities are associated with the ability to host and transmit zoonotic pathogens. To better understand the evolutionary relationship of species-specific glycosylation traits we performed the first systematic ***Phyloglycomics*** study characterising and systematically comparing serum glycome traits from 70 different species of the vertebrate subphylum.

Our study included over 210 individual samples covering 53 mammalian (of these 7 bats), 15 bird, 7 reptile and 2 fish species. Using porous graphitized carbon liquid chromatography coupled to mass spectrometric detection using an ion-trap instrument an in-depth structure characterisation and relative quantitation of the serum glycome traits was achieved. We found that α-Gal epitopes were highly dominating epitopes in Green sea turtles. Many species including birds predominantly used NeuAc to form mono- and disialylated, diantennary N-glycans, though some species such as tortoise opted to not use sialic acid on its serum N-glycans but complex, diantennary N-glycans that are terminated in di- or tri-hexose epitopes that could carry sulfate/phosphate and methylation. Sialic acids were already used by lungfish, mostly as disialylated epitopes attached to di-hexose antenna on complex-type N-glycans. Interestingly, most species except mice and cattle had a clear preference to use NeuAc (>95%) rather than NeuGc when sialic acid was used to cap the non-reducing end of N-glycans. Glyco-traits such as core-fucosylation were just minor components in most species (<10%) except in pigs where almost 50% of serum N-glycans were core-fucosylated. Overall, these data indicate towards the glycome to undergo a parallel evolution as phylogenetically distant species were found to be closely related on a phyloglycome level. We are currently in the process of finalising the largest vertebrate serum N-glycan and tandem MS spectra database, which will be made publicly available or open access through glycobioinformatics platforms such as Glyconnect, GlyCosmos and UniCarbDB respectively.

(P277) *In situ* glycomic analysis of human brains throughout Alzheimer's Disease progression

Tara Hawkinson[1,2], Harrison A. Clarke[1,2], Roberto A. Ribas[1,2], Terrymar Medina[1,2], Damon D. Kooser[1,2], Cameron J. Shedlock[1,2], Stefan Prokop[3,4], Matthew S. Gentry[1,2], Ramon C. Sun[1,2]

[1]Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL 32610, [2]Center of Advanced Spatial Biomolecule Research (CASBR), College of Medicine, University of Florida, Gainesville, FL 32610, [3]Department of Pathology, College of Medicine, University of Florida, Gainesville, FL 32610, [4]Neuromedicine Human Brain and Tissue Bank, College of Medicine, University of Florida, Gainesville, FL 32610,

Glucose hypometabolism is considered a significant and actionable clinical feature of Alzheimer's Disease (AD) and other form neurological disorders. Protein N-linked glycosylation is downstream of glucose central carbon metabolism, and impacts a myriad of intracellular and extracellular processes from adhesion to neuronal signaling cascades. Of particular interest, N-linked glycans are a feature of neuronal homeostasis, including influencing resting membrane potential, axon firing, and synaptic vesicle trafficking. Our lab previously reported a detailed, spatially resolved N-linked glycan analysis within normal and AD, human frontal cortical and hippocampal brain regions suggesting protein hyperglycosylation in the grey matter of AD human brains. Here we expanded on this study with additional 19 human brain tissue samples In collaboration with the Neuromedicine Human Brain and Tissue Bank. All patients were female, and metadata were included with the tissue, including age, Braak status, CERAD status, Thal amyloid staining intensity, and clinical pathologist official diagnoses. In addition, these patients all contained at least one APOE3 allele, with a majority of the patients genotyped as APOE3/3. Using previously published N-linked glycan workflows, we spatially interrogated N-linked glycan heterogeneity within the grey matter of human AD frontal cortex

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tissue slices throughout disease progression (No AD, AD low, AD mid, and AD high). We identified robust, regional-specific N-linked glycan changes associated with AD in humans throughout their disease progression. This was not specific to a single class of N-glycans, instead, core fucosylated, pauci mannose, high mannose, sialylated, and bisecting classes were all robustly increased as the disease progressed. These data suggest N-linked glycan dysregulation could be a metabolic underpinning of neuronal dysregulation leading to AD, and warrant further mechanistic interrogations.

(P278) Structure, anti-SARS-CoV-2, and anticoagulant actions of novel marine sulfated glycans

Vitor Pomin[1], Rohini Dwivedi[1], Seon Beom Kim[1], Antim K. Maurya[1], Marwa Farrag[1], Hoda A. Ahmed[1]

[1]*Department of BioMolecular Sciences, and Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, MS, USA,*

Sulfated glycans such as glycosaminoglycans are key biological components of the cell surface. These glycans can interact and regulate a variety of functional proteins involved in numerous pathophysiological events, including anticoagulation and SARS-CoV-2 infectivity. My group has been dedicating great effort in the last two decades to understand in detail the structural and functional properties of novel marine sulfated glycans (MSGs). Representative of these MSGs are the fucosylated chondroitin sulfate (FucCS), the sulfated fucan (SF), and the sulfated galactan (SG). These molecules have lately been extracted from seaweeds such as the red alga *Botryocladia occidentalis* (Bo), sea cucumbers such as *Isotichopus badionotus* (Ib), *Holothuria floridana* (Hf), *Thyonella gemmata* (Tg), and *Pentacta pygmae* (Pp), and sea urchins such as *Lytechinus variegatus* (Lv). In the last three years, we were able to fully characterize five new structures (PpFucCS, TgFucCS, TgSF, BoSG 1, and BoSG 2), generate the main NMR solution conformation of a SF-derived tetrasaccharide (LvSF) (PDB 7KS6), isolate four previously characterized MSGs (IbSF, IbFucCS, HfSF, and HfFucCS), generate and structurally characterize the oligosaccharides derived from most of these MSGs, and performed anticoagulant and anti-SARS-CoV-2 activities on both native and oligosaccharide derivatives. A multi-faceted set of analytical and biological methods were employed in our studies concerning the *in vitro* activities of the MSGs as inhibitors of the coagulation and the SARS-CoV-2 infectivity. For this latter case, various variants of concern were included in our investigations. From our results, we were able to identify both non-anticoagulant and anticoagulant MSGs with more potent and efficacious anti-SARS-CoV-2 activities than unfractionated heparin, and produce non-anticoagulant oligosaccharides endowed with great anti-SARS-CoV-2 activity derived from highly anticoagulant MSGs. Structural requirements, such as the 3,4-disulfation in branching fucose units of HfFucCS, were also elucidated in our studies. From our research, we were able to prove the efficiency of exploiting the MSGs and low molecular weight derivatives as biochemical or pharmaceutical tools in the science of glycosaminoglycanomics.

(P279) Role of Glycosaminoglycans in Modulation of Lysosomal Proteases Relevant to Alzheimer's Disease

Tamim Chiba[1,2], Nehru Viji Sankaranarayanan[1,2], Umesh R. Desai[1,2]

[1]*Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA, USA,* [2]*Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, VA, USA*

Alzheimer's Disease (AD), a chronic neurodegenerative disorder, and the most common form of dementia is characterized by a loss in memory and other cognitive abilities. At the microscopic level, AD presents with extensive neuronal loss, amyloid plaques, and neurofibrillary tangles. At the molecular level, β-secretase (BACE1) cleaves at the β-site of amyloid precursor protein (APP) to generate amyloid-β, the primary component of amyloid plaques. On the other hand, cathepsin D (catD) has been shown to break down tau tangles and has been isolated with amyloid plaques. The majority of proteins involved in this pathophysiology are known to bind to natural biopolymers called glycosaminoglycans (GAGs), which are primarily composed of either heparin/heparan sulfate (Hp/HS) or chondroitin/dermatan sulfate (CS/DS) type. These highly sulfated biopolymers are located on cell surfaces and in the extracellular matrix of the brain and interact and modulate nearly all proteins involved in AD pathogenesis.

Elucidating fundamental principles involved in GAG recognition and modulation of BACE1 and catD may serve as a starting point to design GAG-based therapeutics. The elementary steps involved in activation of both lysosomal proteases have been studied; however, a lack of consensus regarding their inhibitory and activating properties is lacking. Whereas GAGs have been found to inhibit catD, they have also been found to stimulate the activity of mature catD.

To elucidate the molecular processes following GAG binding to the two lysosomal proteases, we first performed virtual library screening studies followed by biochemical enzyme modulation studies. A library of 140 distinct commercially available GAG sequences of chain length tetra- to nona- saccharide was screened using our in-house combinatorial virtual screening library (CVLS) algorithm. An unbiased method of screening various electropositive surfaces of catD and BACE1 yielded the preferred binding site as well as the preferred mode of binding. The *in silico* binding affinity (parameter=GOLDScore), predicted by CVLS, does not correlate with either chain length or degree of sulfation; however, a distinct pharmacophore comprising

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certain hydroxyl, carboxylate, and sulfate groups could be derived using an *in silico* consistency of binding (parameter=RMSD). Interestingly, both enzymes present two binding sites with varying GAG binding affinities. Our *in vitro* results show that at low GAG concentrations, the enzyme activity increases, whereas higher concentrations induce inhibition of enzyme. This raises a novel possibility that the two putative binding sites may be serving different purposes depending on the level of expression or availability of different GAGs. Overall, the phenomenon of GAG-concentration dependent dual modulation of lysosomal enzymes (activation and inhibition) presents a very interesting molecular regulatory mechanism with possible consequences in clearance of plaques and tangles.

Acknowledgement: We thank the NIH U01 CA241951 and P01 HL151333 for supporting this research (URD).

(P280) *RatMap:* Towards Providing an Organ-Resolved Glycoproteome Map of Wistar Rats

Marc Driessen[1], Ramona Lesch[1], Ulla I.M. Gerling-Driessen[2]

[1]*Heinrich-Heine-University Duesseldorf, Proteome Research, Institute of Molecular Medicine I, Duesseldorf, Germany,* [2]*Heinrich-Heine-University Duesseldorf, Institute of Organic and Macromolecular Chemistry, Duesseldorf, Germany*

Changes in glycosylation are hallmarks of many diseases like, cancer, congenital disorders of glycosylation and even infectious and neurodegenerative diseases. Recently, many glycoproteins as well as overall changes in glycosylation have become a focus in biomarker research as well as basic research, as they hold a wealth of biological information, that is inaccessible via standard, quantitative proteomics approaches. Currently, incorporating glycoproteomic data in biological evaluations is still challenging due to complexity and inaccessibility, especially when analyzing intact glycopeptides. When mapping system wide changes in glycoproteomes, one of the challenges in data analysis and interpretation is the limited access to reliable, comparable and accessible information on the protein level. Database filed information on glycoprotein heterogeneity is limited. This gets more pronounced if one does not consider the human proteome, but rather proteomes of commonly used mammalian animal models like mice and rats (the most used animal for toxicological evaluation). This lack of such information additionally hampers evaluation of biological findings as information on the baseline glycoproteome is needed for assessment. This is especially true for non-specialist labs.

We recently established a glycoproteomics method platform, that we employ to facilitate experiments from a first 'fingerprinting' to a detailed analysis of the glycosylation status of complex biological systems. It is built around an easily transferable set of protocols, that will allow easy lab-to-lab transfer and allow for comparable results. With the "*RatMap*" project, we demonstrate the applicability of our method platform by providing an organ resolved glycoproteome map of Wistar rats. Starting with male animals, we are currently mapping more than 25 separate organs. We plan to focus on providing a robust, achievable glycoproteome coverage, as well as associated protocols, readily transferred to other, non-specialist labs. This will, in the future, allow referencing the baseline glycoproteome to any disease model in Wistar rats. Here, we present the study outline, along with first results, demonstrating the depth of the glycoproteome and glycan heterogeneity coverage.

(P281) Advancing Glycoinformatics Data Integration within CFDE through GlyGen

Jeet Vora[1], Jingyue Wu[1], Daniall Masood[1], Rene Ranzinger[2], Michael Tiemeyer[2], Raja Mazumder[1]

[1]*Department of Biochemistry & Molecular Medicine, The George Washington School of Medicine and Health Sciences, Washington, DC, 20052, USA,* [2]*Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, 30602, USA*

The Common Fund Data Ecosystem (CFDE), an initiative under the NIH is dedicated to addressing the challenges faced by both data users and generators in the realm of Common Fund (CF) data. Its primary objective is to establish a seamlessly interconnected data ecosystem that adheres to the FAIR principles (Findable, Accessible, Interoperable, and Reusable). By accomplishing this, the CFDE strives to simplify accessing and leveraging cross-domain data from a multitude of sources to propel biomedical research forward. In its current undertaking, CFDE has the participation of more than 12 Data Coordinating Centers (DCCs), who formed seven collaborative partnerships aimed at bridging distinct data types and omics data to address a wide spectrum of biomedical challenges.

GlyGen (https://glygen.org) joined CFDE in 2021 as a DCC for glycomics and glycoproteomics data. It actively integrates datasets with the CFDE portal using the Crosscut Metadata Data Model (C2M2) and CFDE submission tools. These datasets cover glycans, proteins, and glycoproteins from various organisms within GlyGen as well as glycan array data. Researchers can easily access and search these datasets via the CFDE portal (https://app.nih-cfde.org/), which also cross-referencing GlyGen data with similar data from other DCCs. As part of CFDE's content generation intiative, GlyGen enhanced the CFDE portal with cross-references to GlyGen data entries and visual elements for glycan structures, aiding users in exploring glycan data via the CFDE portal.

GlyGen also advances glycomics data integration and use through active participation in CFDE partnership projects. It plays a pivotal role in projects such as Data Distillery, Workflow Playbook, Biomarker Data, and COMPA partnership. GlyGen's overarching goal in these partnerships is to foster synergy among gene, protein, biomarker, and glycan information and diverse

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-omic data from various DCC resources, including GTEx, LINCS, 4D Nucleosome, IDG, Metabolomics Workbench, and Kids First. Each partnership addresses specific use cases and challenges:

The Data Distillery utilizes a knowledge graph database (KFDB) to integrate data from participating DCCs to facilitate user-friendly data access and exploration, enabling scientific insights. The Workflow Playbook project constructs workflows by drawing knowledge from various DCCs to support data-driven hypotheses, demonstrating interoperability among different DCC datasets at the API level. The Biomarker Data project focuses on mapping biomarkers across multiple DCCs and biomedical disciplines. This partnership bridges pre-clinical and clinical data, enriching our understanding of the "biomarker genome" within the druggable genome and drug compound space. The Communication and Outreach to Maximize Product Adoption (COMPA) partnership aims to maximize awareness and adoption of CFDE products through market research, outreach, dissemination, and user recruitment for various CFDE products.

(P282) Unique analysis of water behavior around glycans

Tatsuya Moriguchi[1], Yuta Maki[1,2], Ryo Okamoto[1,2], Yasuhiro Kajihara[1,2]

[1]*Department of Chemistry, Graduate School of Science, Osaka University,* [2]*Forefront Research Center, Graduate School of Science, Osaka University*

Glycans of glycoproteins regulate protein functions. However, the clear processes are still unknown. We hypothesize that glycans regulate water behavior around glycoproteins. In order to verify this, we have investigated interaction between sugar and water by a newly established unique system enabling control of amounts of water in an NMR tube. This new system can regulate water amount ranging from over 100 equivalents to one equivalent of water against sugar molecules even under low concentration of sugars. Therefore, this system enabled us to observe and analyze the interaction between glycans and bulk-like water molecules and hydration water molecules. We made a sample in DMSO changing water amount and sugar of interest and then measured NOESY, DOSY, T1, and T2 relaxation in the presence of water ranging from 100 equivalents to 1 equivalent even for dilute sugar samples. As a result, we successfully observed a signal that seems to be sugar hydrated water. We have performed NMR measurements for glucose, galactose, trehalose, and complex type N-glycans. Among these sugars, the T2 relaxation of trehalose and N-glycan remarkably decreased compared to other monosaccharides. In this presentation, we would like to discuss how glycans on glycoproteins control water behavior in detail.

(P283) New avenues for biomarker discovery in human blood plasma via improved in-depth analysis of the low-abundant N-glycoproteome

Frania Jaqueline Zuniga-Banuelos[1,2], Marcus Hoffmann[1], Udo Reichl[1,3], Erdmann Rapp[1,2]

[1]*Bioprocess Engineering, Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany,* [2]*glyXera GmbH, Magdeburg, Germany,* [3]*Bioprocess Engineering, Otto von Guericke University Magdeburg, Magdeburg, Germany,*

To understand implications of protein glycosylation for clinical diagnostic and biopharmaceuticals, innovative glycoproteomic technologies are required. Significant advances were made in the last years, particularly in structure-focused *N*-glycoproteomic analyses. The current mass spectrometric analysis of intact *N*-glycopeptides, employing different fragmentation methods together with glycan oxonium ion profiling, enables to reliably discriminate between different *N*-glycan structures. Still, there are weaknesses that the *N*-glycoproteomics approaches must overcome: 1) handling of incorrect identifications, 2) identification of rare or modified *N*-glycans, and 3) insufficient glycoproteomic coverage, especially in complex samples. To address these limitations, we have developed an innovative *N*-glycoproteomic workflow that aims to provide a comprehensive analysis of structural and site-specific *N*-glycosylation on human blood plasma proteins. The sample preparation workflow includes protein depletion, various fractionation strategies, and the application of high-resolution mass spectrometry with stepped collisional fragmentation. By establishing a decision tree procedure for data validation, our data analysis workflow allowed the detection and re-annotation of incorrect matches caused by, for example, miss-assigned *N*-glycan compositions or the absence of *N*-glycan building blocks in the *N*-glycan database. As a result, we significantly improved the description of the *N*-glycan micro-heterogeneity. We achieved the reliable identification of *N*-glycan modifications (e.g. sulfation), rare *N*-glycans (e.g. with terminal glucuronic acid), and the differentiation of ambiguous *N*-glycan structures, such as multi-antennae versus diLAcNAc or core-fucose versus antenna-fucose. Furthermore, with this workflow, we were able to advance in the analysis of human blood plasma glycoproteins down to concentrations as low as 10 pg/mL. A total of 1929 *N*-glycopeptides and 942 *N*-glycosylation sites derived from 805 human glycoproteins of middle to low abundance were identified. Overall, the presented workflow offers advantages in terms of extending the description of protein *N*-glycosylation and thus promotes the discovery of blood plasma biomarkers.

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(P284) Glycoconjugate data integration using glycan chemical structure extraction software

Masaaki Matsubara[1], Kiyoko F. Aoki-Kinoshita[2], Issaku Yamada[1]

[1]*The Noguchi Institute,* [2]*GalSIC, Soka University*

Various databases have been developed to store glycan-related information. Since most glycans function as glycoconjugates, database integration between different research fields is essential in glycoscience, which requires knowledge across the fields. On the other hand, especially between chemistry and biology, it has not been easy to connect their data due to the large gap between their representations of glycan structures. We have developed WURCS (Web 3.0 Unique Representation of Carbohydrate Structure) as a notation for representing all glycan structures with unique character strings. We have been able to greatly promote glycan-related database integration, especially for biology, by unifying different glycan structure notations, such as GlycoCT and IUPAC, into WURCS. On the other hand, since databases for chemistry contain not only glycans but also many other compounds in the form of structural formulas, we needed a method to extract the glycans in chemical compounds and to integrate them with glycan and glycoconjugate databases. To address this, we have designed original rules to identify glycan structures from structural formulas of compounds and developed software to extract glycans mechanically based on these rules.Currently, we are applying this software to the structures in the LIPID MAPS Structure Database (LMSD) and PubChem in order to extract their glycoconjugate data. As a result of analyzing the data in LMSD, we could confirm that glycans can be extracted appropriately and that the lipid portion and the glycan portion can be clearly distinguished except for few glycosides and glycolipids. In addition, we are currently cooperating with PubChem by providing our software to enable the automated detection of WURCS from compounds, the interconnection with glycan structure databases by WURCS, and functional verification.In this presentation, we will not only show results of these efforts but also discuss what structures should be recognized as monosaccharides and glycans.

(P285) Magnetic beads-based method of plasma N-glycoproteomics toward automated analysis

Kazuki Nakajima[1], Ken Hanzawa[1], Reiko Murakami[1]

[1]*Institute of Glyco-core Research (iGCORE), Gifu University,*

Plasma glycoproteomics holds enormous potential to identify personalized biomarkers for disease diagnosis. A rapid and streamlined workflow is essential for developing a standardized framework for large-scale cohort analysis. Thus, this study aimed to optimize the magnetic bead-based method for automated sample preparation. After removing albumin and IgG, human plasma samples were subjected to trypsin/Lys-C digestion on carboxylate-modified magnetic beads using the SP3 method. The remaining contents were immediately added to a loading solution to remove non-glycopeptides. Subsequently, glycopeptides were enriched by hydrophilic interaction liquid chromatography (HILIC) in neutral conditions. Using LC-MS/MS, 2,024 N-glycoforms were identified from 141 glycoproteins by Byonic. The results were further described by Progenesis in a two-dimensional map illustrating the point with m/z and retention time of each glycopeptide. The reproducibility of our method was evaluated by coefficient of variations in intra-day and inter-day assays. Furthermore, we applied our method to patients with diabetic kidney diseases. Several glycopeptides derived from haptoglobin, kininogen, and immunoglobulin J chain were significantly altered during disease progression and clearly monitored by the glycopeptide map. Thus, this method will enable us to easily perform plasma glycoproteomics on multiple samples for biomarker identification.

(P286) GlyGen: Computational and Informatics Resources for Glycoscience

Rene Ranzinger[1], Jeet Vora[2], Sujeet Kulkarni[1], Karina Martinez[2], Jingyue Wu[2], Mindy Porterfield[1], Robel Kahsay[2], Nathan Edwards[3], Raja Mazumder[2], Michael Tiemeyer[1]

[1]*Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, 30602, USA,* [2]*Department of Biochemistry & Molecular Medicine, The George Washington School of Medicine and Health Sciences, Washington, DC, 20052, USA,* [3]*Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC, 20007, USA,*

Advancing our understanding of the roles that glycosylation plays in development and disease is frequently hindered by the diversity of the data that must be integrated to gain insight into these complex phenomena. GlyGen is an initiative with the goal of democratizing glycoscience research by developing and implementing a data repository that integrates diverse types of data, including glycan structures, glycan biosynthesis enzymes, glycoproteins, along with genomic and proteomic knowledge. To achieve this integration, GlyGen has established international collaborations with database providers from different domains (including but not limited to EBI, NCBI, PDB, and GlyTouCan) and glycoscience data producers. Information from these resources and groups are standardized and cross-linked to allow queries across multiple domains. To facilitate easy access to

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this information, intuitive web-based interfaces (https://glygen.org and https://data.glygen.org) have been developed to visually represent the data and enhance access to the integrated datasets. In addition to the browser-based interface we also developed RESTful webservice-based APIs and a SPARQL endpoint, allowing programmatic access to integrated datasets.

For each glycan and glycoprotein in the dataset, GlyGen provides a details page that displays information from the integrated resources in a concise representation. Individual details pages are interlinked with each other allowing easy data exploration across multiple domains. For example, users can browse from the webpage of a glycosylated protein to the glycan structures that have been described to be attached to this protein, and from there, to other proteins that carry the same glycan. All information accessed through GlyGen is linked back to original sources, allowing users to easily access and browse through information pages in these resources as well. The GlyGen portal itself provides multiple different search interfaces for users to find glycans and proteins based on their properties or annotations. The most advanced version of these searches is the GlyGen Super Search that visualizes the entire data model in one graph and enables users to find glycans and proteins by adding constraints to this graph. Beyond the data on glycans and proteins, GlyGen also provides multiple tools for studying glycosylation pathways, investigating relationships between glycans based on incomplete structures or mapping of different ID namespaces.

Our goal is to provide scientists with an easy way to access the complex information underlying state-of-the-art knowledge that describes the biology of glycans and glycoproteins. To schedule an individual demo of GlyGen or add your data to GlyGen contact Rene Ranzinger (rene@ccrc.uga.edu).

(P287) Hepatic Sulf2 Reduction by Oral 3'Sialyllactose Treatment Improved Lipoprotein Clearance and Lowered Plasma Triglycerides in Hyperlipidemia Model

Chelsea Painter[1,2], Ariane R. Pessentheiner[1,2], Sophia R. Albertelli[1], Lars Bode[2,3], Philip L.S.M. Gordts[1,2]

[1]*Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA,* [2]*Glycobiology Research and Training Center (GRTC), University of California San Diego, La Jolla, CA 92093, USA,* [3]*Department of Pediatrics, Larsson-Rosenquist Foundation Mother-Milk-Infant Center of Research Excellence (MOMI CORE), and Human Milk Institute (HMI), University of California San Diego, La Jolla, CA 92093, USA,*

Cardiovascular diseases (CVDs) are the leading cause of death for people worldwide. Narrowing of the arteries by atherosclerotic plaques is the most common etiology of CVD. Risk factors for atherosclerosis include systemic inflammation and elevated plasma triglyceride and cholesterol levels, or combined hyperlipidemia. Human milk is rich with bioactive compounds such as unconjugated oligosaccharides that exhibit diverse biological functions including regulating development and both innate and adaptive immunity. These individual glycans are being characterized for their distinct bioactivity and effects on biological systems. Preliminary research demonstrates that the oral administration of HMO 3'Sialyllactose (3'SL) reduces atherosclerotic lesion volume and plasma lipid levels in a hyperlipidemia model. While it is known that absorbed HMOs can have systemic effects, the mechanism behind the mitigation of hyperlipidemia by 3'SL is unknown. We evaluated factors that affect plasma lipid levels such as lipid absorption in the gut, lipid lipolysis and uptake in peripheral tissues, de novo triglyceride-rich lipoprotein (TRL) production, and TRL clearance in the liver. While other factors displayed little to no differences between groups, we found a significant increase in the heparan sulfate proteoglycan Syndecan-1 (SDC1)-mediated hepatic TRL clearance in 3'SL-treated animals. RNA-seq analysis of the liver revealed a reduction in hepatic Sulfatase-2 (*Sulf2*) in the 3'SL-treated animals. SULF2, a de-sulfating enzyme of HS chains, has been previously shown to decrease hepatic Syndecan-1 mediated TRL clearance. Antisense knockdown of *Sulf2* in combination with 3'SL treatment in our in vivo hyperlipidemia model demonstrated a lack of additive TRL lowering confirming that 3'SL lipid lowering is mediated through a reduction of liver SULF2. These studies begin to elucidate the mechanism of action for 3'SL in reducing hyperlipidemia in mouse models and provide evidence towards the use of HMOs in treating human diseases.

(P288) Chemoenzymatic approach to Proteoglycan mimetics for investigating precise Heparan Sulfate interactions

Pravinkumar Choudhary[1], Julianna Follmar[1], Hancy Barnes[2], Alexander Parmele[1], Philip Gordts[2,3], Kamil Godula[1,3]

[1]*Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0358, United States,* [2]*Department of Medicine, Division of Endocrinology and Metabolism, University of California San Diego, 9500 Gilman Drive, La Jolla California 92093-0687, United States,* [3]*Glycobiology Research and Training Center, University of California San Diego, 9500 Gilman Drive, La Jolla California 92093-0687, United States,*

Glycocalyx is a complex mixture of components which creates a carbohydrate-rich layer that coats the surface of cells and plays a crucial role in cell biology and physiology. Proteoglycans (PGs) are ubiquitous molecules that function as critical components of the extracellular matrix and are responsible in regulating many signaling proteins. PGs are composed of linear chains of glycans that are covalently attached to a protein core. Heparan Sulfate (HS), a class of PGs, participate and regulate various cellular functions including cell adhesion, proliferation, migration, survival, death stem cells differentiation and growth factors signaling.

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In vitro studies of HSPGs are essential for understanding the biological functions, interactions, and signaling pathways. Due to structural complexity, chemical variability, HSPGs are difficult to isolate and chemically synthesize PGs limits its in vitro studies. To overcome these challenges, we have developed an improved method to control valency and variability of PG structures using chemoenzymatic approach. The resulting streptavidin-based bioconjugates enable the attachment of a wide range of biotinylated targets, including biotinylated antibodies, membrane virions, liposomes, RNA, DNA, fatty acids, and fluorescent probes. These versatile bioconjugates can be utilized in diverse applications to probe and modulate Heparan Sulfate protein interactions at the cell surface.

(P289) Structural requirements in anticoagulant and anti-SARS-COV-2 activities of holothurian fucosylated chondroitin sulfate oligosaccharides

Marwa Farrag[1,2], Rohini Dwivedi[1], Poonam Sharma[3], Deepak Kumar[3], Ritesh Tandon[1,3,4], Vitor Pomin[1,5]

[1]*Department of BioMolecular Sciences, University of Mississippi, Oxford, Mississippi, USA,* [2]*Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt,* [3]*Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, Mississippi, USA,* [4]*Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi, USA,* [5]*Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, MS, USA,*

Fucosylated chondroitin sulfate (FucCS) is a unique glycosaminoglycan found primarily in sea cucumbers. This marine sulfated glycan is composed of a chondroitin sulfate backbone decorated with fucosyl branches attached to the glucuronic acid. FucCS has been shown to be effective against severe acute respiratory syndrome coronavirus (SARS-CoV-2). These molecules are also endowed with potential anticoagulant effects, and this can lead to bleeding risks when explored as antivirals. Previous studies have suggested that molecular weight reduction of these glycans through depolymerization may offer an effective way to gain a selective anti-SARS-CoV-2 activity while minimizing the associated anticoagulant effects. In this work, we aim to generate oligosaccharides of the FucCS from *Holothuria floridana* (HfFucCS) and investigate its potential inhibitory properties against blood clotting and SARS-CoV-2 infection. The HfFucCS oligosaccharides were prepared by free-radical depolymerization using copper-based Fenton reaction. The derived oligosaccharides were assessed by a variety of analytical techniques, including 1D ^{1}H nuclear magnetic resonance spectroscopy, polyacrylamide gel electrophoresis, and size-exclusion chromatography. Activated partial thromboplastin time and assays using protease (factors Xa and IIa) and serine protease inhibitors (antithrombin, and heparin cofactor II) in the presence of the sulfated carbohydrates were used to monitor anticoagulation. Anti-SARS-CoV-2 effects were measured using the concentration–response inhibitory curves of HEK-293T-human angiotensin-converting enzyme-2 cells infected with a baculovirus pseudotyped SARS-CoV-2 wild-type and delta variant spike (S)-proteins. The cytotoxicity of native HfFucCS and its oligosaccharides was also assessed. In our study, we were able to generate one HfFucCS oligosaccharide fraction devoid of high anticoagulant effect but still retaining considerable anti-SARS-CoV-2 actions against both variants.

(P290) Competitive Effect of Marine Sulfated Glycans on Thrombin Binding to Surface Heparin by Surface Plasmon Resonance

Hoda Ahmed[1,2], Vitor Pomin[1,3]

[1]*Department of BioMolecular Sciences, University of Mississippi, Oxford, Mississippi, USA,* [2]*Department of Pharmacognosy, School of Pharmacy, Al-Azhar University, Nasr-City, Cairo, Egypt,* [3]*Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, Mississippi, USA,*

Marine organisms are a rich source of biologically active compounds, including unique sulfated glycans endowed with potential medical applications. The marine sulfated glycans (MSGs) show unique structures composed of defined oligosaccharide building blocks and specific biological effects such as those seen in clotting inhibition. The anticoagulant effect of MSGs depends ultimately on certain structural features such as molecular weight, monosaccharide composition, glycosidic linkage, sulfation pattern and levels of fucosylation. Sulfated fucan (SF), sulfated galactan (SG), and fucosylated chondroitin sulfate (FucCS), are classes of MSGs widely used in research. The MSGs used for this research were extracted from one red alga: *Botryocladia occidentalis* (Bo), one sea urchin: *Lytechinus variegatus* (Lv), and four sea cucumbers: *Isostichopus badionotus* (Ib), *Holothuria floridana* (Hf), *Pentacta pygmaea* (Pp), and *Thyonella gemmata* (Tg). In this work, we aim to test the competitive inhibitory effects of 4 FucCS (IbFucCS, HfFucCS, PpFucCS, and TgFucCS), 4 SF (IbSF, HfSF, TgSF, and LvSF) and 2 SG (BoSG-1 and BoSG-2) on the binding interaction of the main coagulation factor, thrombin (IIa), to surface heparin using surface plasmon resonance (SPR). Factor IIa was mixed with different concentrations of unfractionated heparin (UFH), low molecular weight heparin (LMWH) and the 10 MSGs, and then injected over a heparin sensor chip. IC50 values reflecting the concentration of the competing analyte resulting in a 50% decrease in protein binding, measured in response units (RUs), were calculated from the RU plots as a function of sulfated glycan concentration. Our results showed that the dissociation constant of IIa binding to surface heparin was 7.5×10^{-8} M, and the IC_{50} of UFH and LMWH were respectively 9.1 × 10–8 M and 4.57 × 10–6

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M. Preliminary data showed that TgSF, and BoSG-2 were comparable to UFH effect on binding of factor IIa to surface heparin given the same order of magnitude (10^{-8} M range) of their measured IC_{50} values. The IC_{50} of IbFucCS, IbSF, BoSG-1, HfSF, TgFucCS, and LvSF showed more moderate effect (10^{-7} M range), while HfFucCS and PpFucCS showed lower effect similar to LMWH (10^{-6} M range). All measurements were obtained in triplicate for significant statistical interpretation. Although the anticoagulant action of MSGs has been known and studied in the last two decades, this work is the first one comprising a systematic analysis of a large collection of MSGs of defined chemical structures in interaction with IIa. This SPR-based study will ultimately unveil the structural requirements regarding the IIa binding properties of MSGs in their anticoagulant process.

(P291) Functional Annotation of Glycan Motifs via Glycosylation Enzymes and the GlyGen GlycoTree Sandbox

Doroteja Dragovic[1], Nathan Edwards[1]

[1]*Clinical and Translational Glycoscience Research Center, Georgetown University, Washington, D.C.,*

The GlyGen GlycoMotif data-resource integrates nearly 700 glycan motifs and determinants collected from eleven different glycoinformatics resources, provides precomputed semantic alignments with GlyTouCan glycan structures, and hosts the actively curated set of glycan motifs for the GlyGen glycan knowledge-base. Despite glycans' significant structural heterogeneity, recurring glycan motifs and determinants are understood to be responsible for driving glycans' cell- and protein- binding activity and thus their functional role in specific biological contexts. We seek to explore the inference glycan motif function based on the phenotypes, cell-types, and other functional annotations associated with the glycoenzymes required for synthesis of glycans containing a motif.The GlyGen GlycoTree Sandbox associates human and mouse glycotransferases with the monosaccharide residues of GlyGen N-linked and O-linked glycan structures. GlycoMotif motif alignments to corresponding GlyGen structures make it possible to map Sandbox glycotransferases on structures' residues to the corresponding residue of the motif. Specific motif resides may be annotated repeatedly by the same enzymes or by different enzymes by virtue of motif alignments to a variety of structures and multiple placements on a single structure. A key computational innovation necessary to transfer GlycoTree Sandbox enzyme annotations is the use of canonical monosaccharide ids, and motif-to-structure and structure-to-structure alignments that identify corresponding residues. For structure-to-structure alignments a single bijection between the structures' monosaccharides is returned, while for motif-to-structure alignments, pairs of corresponding residues for all possible placements of the motif on the structure are computed. The GlycoMotif glycan motif data-resource integrates glycoenzyme annotations with each motif, and provides enzymes pages for each glycoenzyme showing the motifs associated with at least one structure they help to synthesize. Enzymes are annotated with genes names, UniProt and MGI accessions, and links to functional annotation resources such as the International Mouse Phenotype Consortium (IMPC). Human glycoenzymes implicated in congenital disorders of glycosylation (Freeze, et al., 2014) are also shown. Other functional annotations associated with genes, notably mined from large-scale gene-expression data-resources such as the Genotype-Tissue Expression Project (GTex), suggest tissue and/or cell-type specific expression of glycoenzymes, and may suggest such specificity for glycan structures containing specific glycan motifs.

(P292) A central regulator of blood cell production and function is the sialyltransferase ST6GAL1

Joseph T.Y. Lau[1]

[1]*Department of Molecular and Cellular Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA,*

Maintaining effective production of blood cells throughout the lifetime of an animal is essential. The sialyltransferase ST6GAL1 mediates the α2,6-sialylation of Gal(β1,4)GlcNAc glycan termini typically on N-linked glycans. ST6GAL1 typically resides in the intracellular ER-Golgi secretory complex where it glycosylates complex glycoconjugates in biosynthetic transit. Significant levels of ST6GAL1 are present also in the extracellular milieu such as the systemic circulation. Extracellular ST6GAL1 drives B cell production but attenuates granulopoiesis. We show that extracellular ST6GAL1 promotes hematopoietic recovery following acute injury such as iatrogenic, accidental, or malicious radiation exposure. Animals lacking in functional ST6GAL1 suffer greater injury with delayed recovery times. In extreme cases of acute radiation syndrome (ARS) mortality results. Absence of ST6GAL1 results in mortality at lower exposure dosages that are related to incapacitating stem cells to rejuvenate the functional tissues. Addition of recombinant ST6GAL1 to the extracellular pool rejuvenates the immediate ability to rejuvenate hematopoietic progenitors *ex vivo*. We also present data supporting the existence of extracellular co-factors that direct and shape extracellular ST6GAL1 action. Taken together, the data point to the concept that ST6GAL1, with the potential to act in endocrine, paracrine, or autocrine modes, is a regulator of multiple aspects of hematopoiesis and blood cell function.

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