Session 1: Glycopathologies and Therapeutics

(Key1-001) Congenital Disorders of Glycosylation: Glycobiology at the Bedside

Andrew C. Edmondson[1]

[1]*Children's Hospital of Philadelphia, University of Pennsylvania,*

Congenital disorders of glycosylation (CDG) are a group of rare monogenic human disorders caused by defects in the genes encoding the proteins that generate, attach, and modify glycans, thus disrupting cellular glycosylation machinery. Over 200 CDG caused by disruptions of 189 different genes are currently known. The multi-system disease manifestations of the CDG disorders highlight the importance of glycosylation across the organ systems. Clinical manifestations of CDG tend to group among genes contributing to the same glycosylation pathways, suggesting shared pathophysiology related to the glycosylation disruptions. However, the underlying glycosylation disruptions and pathophysiologic mechanisms responsible for specific CDG clinical manifestations have been determined for only a few hypoglycosylated proteins.

The Frontiers in CDG Consortium (FCDGC) is an international network of clinical sites, laboratories, and patient advocacy groups established in 2019 to improve clinical symptoms, quality of life, and life expectancy for individuals with CDG. FCDGC seeks to answer decades of unresolved questions, address knowledge gaps, develop and validate new biochemical diagnostic techniques and therapeutic biomarkers, and explore novel therapeutic options for CDG. Over the past 5 years, FCDGC has launched a Natural History Study with over 300 CDG patients, discovered novel biomarkers suggesting new mechanisms of disease, and launched clinical trials aiming to restore appropriate glycosylation and targeting newly identified potential mechanisms of disease.

Technical advances in glycobiology are making it increasingly possible to comprehensively catalog glycoproteomic data and to probe functional impact of altered glycosylation. My laboratory applies glycoproteomic technologies to samples from human subjects and genetic model systems to identify glycosylation abnormalities and unlock new insights from translational glycobiology. Current findings and accomplishments highlight the ongoing bottlenecks and knowledge gaps at intersections of glycobiology and clinical care requiring further investigation.

(Key1-002) Toward effective gene therapy for classic galactosemia

Shauna A. Rasmussen[1], Olivia Garrett[1], Ingrid Smith[2], Ashlyn Farnham[2], Judith L. Fridovich-Keil[1]

[1]*Department of Human Genetics, Emory University School of Medicine,* [2]*Emory College, Atlanta GA 30322 USA,*

Classic galactosemia (CG) results from profound deficiency of galactose-1-P uridylyltransferase (GALT) and is identified by newborn screening in close to 80 infants born each year in the US. Spared the potentially lethal acute symptoms of disease by early detection with immediate and life-long dietary restriction of galactose, by elementary school most children with CG have nonetheless grown to experience a range of developmental delays and other problems. The mechanisms underlying these long-term complications remain unknown, including which, if any, of the galactose metabolites that accumulate in blood and tissues is or are causal. In light of these uncertainties, and mindful that GALT is not lysosomal and therefore not easily targeted for enzyme replacement with uptake from the bloodstream via the mannose-6-phosphate pathway, we are testing the efficacy and duration of systemic GALT gene therapy using AAV in a GALT-null rat model of CG. Specifically, we are working to define how much GALT restoration is required, in what proportion of cells, in which tissues, and when in development to achieve meaningful and long lasting metabolic and phenotypic rescue. Further, we are testing the potential benefit of targeting virus-encoded GALT mRNA and protein transgene products to extracellular vesicles to facilitate both local and systemic distribution of GALT from cells that carry the recombinant viral genome to cells that do not. The results of this work should help to inform future interventions for CG, and potentially other disorders, including a range of congenital disorders of glycosylation.

(Key1-003) Dolichol Synthesis: From a Human Disease to Novel Twists and Required Detours

Matthew P. Wilson[1,†], Takfarinas Kentache[2,3,†], Charlotte R. Althoff[1,4,†], Céline Schulz[4], Geoffroy de Bettignies[4], Gisèle Mateu-Cabrera[1], Loreta Cimbalistiene[5], Birute Burnyte[5], Grace Yoon[6,7,8], Gregory Costain[6,8,9,10], Sandrine Vuillaumier-Barrot[11], David Cheillan[12], Daisy Rymen[13], Lucie Rychtarova[14], Hana Hansikova[14], Marina Bury[2,3], Joseph P. Dewulf[2,3], Francesco Caligiore[2,3], Jaak Jaeken[13], Vincent Cantagrel[15], Emile Van Schaftingen[2,3,†,‡], Gert Matthijs[1,†,‡], François Foulquier[4,†,‡], Guido T. Bommer[2,3,16,†,‡]

[1]Laboratory for Molecular Diagnosis, Center for Human Genetics, KU Leuven, Leuven, Belgium, [2]Metabolic Research Group, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium, [3]WELBIO department, WEL Research Institute, avenue Pasteur, 6, 1300 Wavre, Belgium, [4]Univ. Lille, CNRS, UMR 8576 – UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France, [5]Institute of Biomedical Sciences, Faculty of Medicine, Vilnius University, Vilnius, Lithuania, [6]Division of Clinical and Metabolic Genetics, Hospital for Sick Children, Toronto, Canada, [7]Division of Neurology, Hospital for Sick Children, Toronto, Canada, [8]Department of Paediatrics, University of Toronto, Toronto, Canada, [9]Program in Genetics and Genome Biology, SickKids Research Institute, Toronto, Canada, [10]Department of Molecular Genetics, University of Toronto, Toronto, Canada, [11]AP-HP, Biochimie Métabolique et Cellulaire and Département de Génétique, Hôpital Bichat-Claude Bernard, and Université de Paris, Faculté de Médecine Xavier Bichat, INSERM U1149, CRI, Paris, France, [12]Service Biochimie et Biologie Moléculaire – Hospices Civils de Lyon; Laboratoire Carmen - Inserm U1060, INRAE UMR1397, Université Claude Bernard Lyon 1, Lyon, France, [13]Department of Pediatrics, Center for Metabolic Diseases, University Hospitals Leuven, Leuven, Belgium, [14]Laboratory for Study of Mitochondrial Disorders, Department of Paediatrics and Inherited Metabolic Disorders, First Faculty of Medicine and General University Hospital in Prague, Charles University, Prague, Czechia, [15]Developmental Brain Disorders Laboratory, Université Paris Cité, INSERM UMR1163, Imagine Institute, Paris, France,

†Equally contributing first authors
‡Equally contributing last authors

Dolichol is a lipid critical for N-glycosylation as a carrier for activated sugars and nascent oligosaccharides. It is commonly thought to be directly produced from polyprenol by the enzyme SRD5A3. Instead, we found that dolichol synthesis requires a three-step detour involving additional metabolites, where SRD5A3 catalyzes only the second reaction. The first and third steps are performed by DHRSX, whose gene resides on the pseudoautosomal regions of the X and Y chromosomes. Accordingly, we report a pseudoautosomal-recessive disease presenting as a Congenital Disorder of Glycosylation in patients with missense variants in *DHRSX* (DHRSX-CDG). Of note, DHRSX has a unique dual substrate and cofactor specificity, allowing it to act as a NAD^+-dependent dehydrogenase and as a NADPH-dependent reductase in two non-consecutive steps. Thus, our work reveals unexpected complexity in the terminal steps of dolichol biosynthesis. Furthermore, we provide insights into the mechanism by which dolichol metabolism defects contribute to disease.

(Key1-004) Cipaglucosidase alfa plus miglustat: linking mechanism of action to clinical outcomes in late-onset Pompe disease

Barry J. Byrne[1], Giancarlo Parenti[2], Benedikt Schoser[3], Ans T. van der Ploeg[4], Hung Do[5], Brian Fox[6], Mitchell Goldman[6], Franklin K. Johnson[6], Jia Kang[7], Nickita Mehta[6], John Mondick[8], **M. Osman Sheikh**[6], Sheela Sitaraman Das[6], Steven Tuske[6], Jon Brudvig[6], Jill M. Weimer[6], Tahseen Mozaffar[9]

[1]University of Florida, Gainesville, FL, United States, [2]Metabolic Unit, Department of Translational Medical Sciences, University of Naples Federico II, Naples, Italy, and Telethon Institute of Genetics and Medicine, Pozzuoli, Italy, [3]Friedrich-Baur-Institute, Department of Neurology, LMU University Hospital, LMU Munich, Munich, Germany, [4]Erasmus MC University Medical Center, Rotterdam, Netherlands, [5]M6P-Therapeutics, St Louis, MO, United States, [6]Amicus Therapeutics, Inc., Princeton, NJ, United States, [7]Metrum Research Group, Tariffville, CT, United States, [8]A2-Ai LLC, Ann Arbor, MI, United States, [9]Department of Neurology, University of California, Irvine, CA, United States,

Enzyme replacement therapy (ERT) is the only approved disease-modifying treatment approach for Pompe disease, a rare, inherited metabolic disorder caused by a deficiency in the acid α-glucosidase (GAA) enzyme that catabolizes lysosomal glycogen. The first-generation recombinant human GAA (rhGAA) ERT, known as alglucosidase alfa, can slow muscle degeneration typical of the disease. However, its effectiveness is very limited and often diminishes over time, likely due to poor uptake into target tissues. Next-generation ERTs aim to improve this by increasing the presence of bis-phosphorylated high mannose (bis-M6P) N-glycans on rhGAA, which have a high receptor binding affinity at low interstitial enzyme concentrations, facilitating uptake by the mannose 6-phosphate receptor on target cells. Some methods, however, add bis-M6P to rhGAA using non-natural linkages that human enzymes cannot break down, hindering the endolysosomal glycan trimming that is necessary for full enzyme activation after cell uptake. Furthermore, rhGAA ERTs face potential inactivation during intravenous delivery (and subsequent non-productive clearance) as GAA is an acid hydrolase that is rapidly denatured in the near-neutral pH of the blood. Cipaglucosidase alfa plus miglustat is hypothesized to address these challenges by combining an enzyme enriched with naturally occurring bis-M6P N-glycans with a small-molecule stabilizer. We examine this hypothesis by analyzing data related to the mechanism of action of the enzyme and stabilizer molecule. The extensive *in vitro*, preclinical, and clinical data suggest that cipaglucosidase alfa plus miglustat effectively addresses these challenges, offering significant benefits in pharmacokinetic exposure, target-cell uptake, endolysosomal processing, and clinical outcomes.

(PT-001) Exploring O-Glycobiomarker in Osteoarthritis: Lubricin glycoforms in plasma and synovial fluid

Niclas G. Karlsson (Presenting author)[1], Ali Reza Afshari[1], Vincent Chang[2], Kristina A. Thomsson[3], Jennifer Höglund[3], Ellie Browne[2], George Karadzhov[2], Keira E. Mahoney[2], Taryn M. Lucas[2], Henrik Ryberg[4], Kamlesh Gidwani[5], Kim Pettersson[5], Ola Rolfson[6], Lena I. Björkman[7], Thomas Eisler[8], Tannin A. Schmidt[9], Gregory D. Jay[10], Stacy A. Malaker[2]

[1]Department of Biomedicine and Pharmacy, Faculty of Life Sciences and Health, Oslo Metropolitan University, Oslo, Norway, [2]Department of Chemistry, Yale University, New Haven CT, USA, [3]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [4]Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden, [5]Department of Life Technologies and FICAN West Cancer Centre, University of Turku, Turku, Finland, [6]Department of Orthopaedics, Institute of Clinical Sciences, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [7]Department of Rheumatology and Inflammation Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [8]Department of Clinical Sciences, Danderyd Hospital, Karolinska Institutet, Stockholm, Sweden, [9]Biomedical Engineering Department, University of Connecticut Health Centre, Farmington, CT, USA, [10]Department of Emergency Medicine, Warren Alpert Medical School and Division of Biomedical Engineering, School of Engineering, Brown University, Providence, RI, USA,

Lubricin is a mucin-domain glycoprotein that is crucial for joint lubrication and synovial homeostasis. Given that arthropathies such as osteoarthritis (OA) and rheumatoid arthritis (RA) are concomitant with altered lubricin glycosylation in synovial fluid (SF), we investigated if glycoforms of lubricin also could be detected in OA patients' plasma, reflecting the health status of the OA joint.

We used a combined glycomic/glycoproteomic approach to characterize the glycosylation of lubricin in synovial fluid and plasma. An improved mucin-selective enrichment strategy which employs a catalytically inactive mucinase (StcE) conjugated to solid support allowed us to obtain glycoform specific glycoproteomic data from the low abundant lubricin present in OA patients plasma (< 10 mg/mL). The combined glycomic and glycoproteomic data allowed us to design a sensitive lectin assay to screen for differences in plasma lubricin glycoforms from a biobank of late stage OA patients and controls (224 individuals).

Using glycoproteomics, we obtained near-complete sequence coverage of lubricin, along with many O-glycopeptides from other mucin proteins. We observed a significant amount of truncated O-glycans in SF lubricin from OA patients (47% T-antigen, 35% Tn antigen, and only 18% Sialyl T antigen), which agreed with the glycomic result of SF-associated lubricin. In OA plasma, however, we tended to observe more sialylated O-glycan structures, only 6% T antigen and 2% Tn antigen, while 86% was found as Sialyl T antigen, and 6 % as Disialyl T antigen. From these results we choose five different lectins; Macrophage Galactose-type lectin (MGL), *Sambucus Nigra* Agglutinin (SNA), *Maackia Amurensis* Agglutinin (MAA), Peanut Agglutinin (PNA), and Galectin-3 (Gal-3), to cover the full extent of lubricin glycosylation in plasma and SF, and we developed a Fluorescent Immuno-Lectin Assay (FILA) to measure the levels of lubricin glycoforms in plasma/SF and their potential as biomarkers for disease using our biobank. In OA patients we found decreased level in plasma of SNA binding lubricin ($p = 0.0023$) compared to controls, which suggests less a2,6 linked sialic acid. In addition, we found that lubricin glycoforms correlated both with Body Mass Index (BMI) and age, especially regarding sialylation as measured by both MAA and SNA.

Our data suggest that glycosylation of lubricin is different comparing SF with plasma. Moreover, the glycosylation of plasma lubricin is altered in OA patients compared to controls. We were also able to attribute the difference in SF and plasma lubricin glycosylation to the presence SF and plasma specific lubricin spliceforms, where the majority of lubricin was due to biosynthesis in liver rather than originating from the joint. Future efforts are being directed towards quantification of the glycosylation of the various splice forms present in plasma.

(PT-003) Leveraging zebrafish to study glycosyltransferases mutated in the dystroglycanopathies

M. Chiara Manzini[1], Kyle P. Flannery[1], Brittany F. Karas[1], Shorbon Mowla[1], Kristin R. Terez[1], Namarata Battula[1], Deze Liu[2], Daniel Burbano-Lombana[2]

[1]Rutgers Robert Wood Johnson Medical School and Child Health Institute of New Jersey, [2]Rutgers School of Engineering,

Congenital muscular dystrophies (CMDs) are autosomal recessive disorders characterized by infantile hypotonia and progressive muscle disease. The most severe forms of CMD, the dystroglycanopathies, are also associated with heterogeneous brain and ocular malformations. Dystroglycanopathies are caused by mutations in several glycosyltransferases that converge on the synthesis of a specialized glycan on a-dystroglycan (a-DG), a transmembrane protein required for tissue differentiation and maintenance. Mouse knock-out (KO) models for dystroglycan itself and for the enzymes involved in the initial steps of glycosylation lead to early embryonic or perinatal lethality requiring conditional removal to study specific tissues. Our long-term goal is to leverage the power of the zebrafish as a model of muscle, eye, and brain disease to study genetic and phenotypic variability in different genetic causes of dystroglycanopathy. Here, we introduce two zebrafish KO lines for enzymes that initiate a-DG O-mannosylation, *protein O-mannosyltransferase 1 (pomt1)* and *protein O-linked N-acetylglucosaminyltransferase 2*

(pomgnt2). Previous work showed extreme phenotypic variability in dystroglycanopathy models with some zebrafish mutants showing larval phenotypes and early mortality and others surviving to adulthood with only retinal deficits. We reconcile these findings by showing the critical role of maternal mRNA in masking developmental phenotypes in dystroglycanopathy gene KOs by retaining a-DG glycosylation. Both *pomt1* and *pomgnt2* mRNAs are provided by the mother in the oocyte, and *pomt1* and *pomgnt2* KO larvae generated from heterozygous crosses (termed KO^{Het}) retain glycosylated a-DG during the first weeks of life. Both KO^{Het} lines exhibit prolonged survival with juvenile- to adult-onset muscle disease and locomotor deficits. In contrast, when maternal *pomt1* or *pomgnt2* is depleted by breeding KO^{Het} females, the resulting KOs (termed KO^{KO}s) exhibit profound, larval-onset muscle, brain, and eye phenotypes and reduced survival. RNA-seq studies on larvae showed minimal differential expression between KO^{Het}s and controls, while *pomt1* KO^{KO}s have downregulation of genes involved in muscle formation and contraction. We are further defining novel measures of locomotor function to determine the effect of therapeutic approaches on the muscle. We conclude that consideration of maternal compensation is an essential aspect of modeling dystroglycanopathies in zebrafish. These new models can now be used to study disease progression and novel therapeutic interventions.

(PT-005) A Murine Mpi mutation reproduces aspects of Inflammatory Bowel Disease with the specified role of MUC2 in the progression of the disease

Avishek Roy[1], Steve Meregini[1], Zhenglan Chen[1], Tandav Argula[1], Bruce Beutler[2], Jeffrey A. SoRelle[1,2,*]

[1]*Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX,* [2]*Center for Genetics of Host Defense, University of Texas Southwestern Medical Center, Dallas, TX,*

Rationale: A spontaneous, hypomorphic mutation in *Mpi* created a phenotype which replicates features of Congenital Disorder of Glycosylation-1B (MPI-CDG) including spontaneous gastrointestinal disease. We have generated an Intestinal specific KO of $Mpi^{DVillin}$ to investigate how N-glycosylation protects from inflammatory bowel disease progression and management.

Methods: Phenotypic and histological studies were performed to assess intestinal pathology. Cellular, biochemical, proteomics and immunological studies were performed to determine the effect of glycosylation in intestinal homeostasis (MUC2, ER stress, Goblet cell transcription factor expression, inflammatory cytokines, O and N-glycan analysis by IHC and Mass Spectrometry, bacterial invasion by 16S FISH, microbiome sequencing, and electron microscopy).

Results: $Mpi^{DVillin}$ mice had spontaneous colitis with fewer goblet cells resulting in less mucus (Muc2) secretion. ER stress is high and localized to epithelial cells with higher pro-inflammatory cytokines but lower goblet cell markers. Loss of intestinal mucus barrier significantly disrupts microbiome homeostasis and removes the barrier to bacterial invasion. Loss of Muc2 N-glycosylation was also found to be essential for O-glycosylation of this glycoprotein. Proteomics data revealed depletion of metabolic genes (mitochondrial and fatty acid synthesis) in both large and small intestines. Mannose treatment corrected the phenotypes and furthermore was protective against DSS-induced colitis, even when given after DSS-colitis was started. Mannose alone induced 50-fold higher transcription of goblet cell genes, four-fold higher mucus thickness, and Muc2 levels. These unexpected results may have translational value not-only for MPI-CDG but also inflammatory bowel disorders broadly.

Conclusions: N-glycosylation maintains intestinal homeostasis through mucus secretion by goblet cells. $Mpi^{DVillin}$ mice produce hypoglycosylated, immature mucus causing goblet cell dysfunction. Mannose could be used to prevent or ameliorate inflammatory bowel conditions by increasing mucus and goblet cell production.

(PT-007) Sugar Genes: Biomarkers beneath cancer's glycan patterns open new avenues for diagnostics and treatment

Jing Kai[1], Luyao Yang[2], Yossef De Los Santos[1], Asma S. AlAmoodi[1], Ayman F. AbuElela[1], Alyaa M. Abdel-Haleem[2], Gloria Lopez Madrigal[1], Abdulghani A. Bin Nafisah[3], AlFadel M. AlShaibani[4], Ali S. Alzahrani[3], Vincenzo Lagani[1], David Gomez-Cabrero[1], Xin Gao[2], **Jasmeen S. Merzaban**[1,*]

[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 Centre, Riyadh, 11211, Saudi Arabia,* [4]*Oncology Center, King Faisal Specialist Hospital and Research Centre, Riyadh, 11211, Saudi Arabia,*

*Corresponding author and presenting author

It has been well-demonstrated in glycobiology that as cancer progresses, the patterns that glycans take on the cell surface change in distinct and perceptible ways. Less understood is what goes on at the genetic level to cause these changes in surface sugar

structures. What if there are also identifiable gene-level patterns underlying different cancers? Would this open the way for developing gene therapies and prognostic tools focused on glycosyltransferases (GTs)?

Our research shows that GT genes are indeed impressive biomarkers for cancer because every variety of the disease, from the breast to the brain, has a unique and highly precise GT signature pattern, each distinguishable from the other even at the cancer subtype level. We found that a subset of up to 71 GTs are characteristically present across cancer types as the disease progresses, manifesting in ways particular to each variety. Using machine learning on tissue samples from the Cancer Genome Atlas, we were able to cluster 27 different cancers based on these unique GT expressions with 98% accuracy. The models we developed and verified with independent data sets and patient samples are able to distinguish cancer from normal tissue, cancer types from each other, and cancer subtypes. In other words, we discovered a kind of molecular-level biometric identification for each cancer variety.

This knowledge has invaluable potential for prognosis and therapy. As an example, we developed a breast cancer classifier using 48 GTs that is almost twice as effective as the widely used subtyping kit, PAM-50, at diagnosing Luminal A and B, Basal-like and HER-2 breast cancer from unlabelled tissue. In a separate project involving virtual screening of smart libraries, we have identified small molecules that inhibit the four most differentially expressed breast cancer GTs and are testing the potential of these for clinical application.

In another example of how GT genes can serve as powerful biomarkers, in this case for leukemia, we are targeting the two GTs that cause the creation of the glycan ligand sialyl Lewis X, which renders these cancer cells an adhesive quality that both guides them to the bone marrow and protects them from treatment. Using virtual screening, we identified a number of small molecules that either act as inhibitors blocking leukemic cell bone marrow engraftment or as activators that can be used to treat therapeutic stem cells to facilitate their delivery to the bone marrow. Taken together, our findings demonstrate that glycan genes offer a degree of functional precision that lend them to being powerful biomarkers, ones that future translational medicine approaches cannot afford to ignore.

(P-033) AGEs-RAGE signaling: a potential unifying mechanism linking diabetes and neurodegeneration

Mariyam Khalid[1,2], Carsten Wolff[1], Abdu Adem[2]

[1]Biotechnology Research Center, Technology Innovation Institute, Abu Dhabi, United Arab Emirates, [2]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 reported to contribute to pathogenesis of various complications associated with this disease, including neurodegeneration. In individuals with prolonged diabetes, increased AGEs in the brain upregulate the receptor for AGEs, known as RAGE through a positive feedback mechanism. The predominant feature shared by most neurodegenerative diseases is a common pathological hallmark, the accumulation of characteristic disease-related proteins into insoluble aggregates in vulnerable neurons and glial cells facilitated by processes like misfolding, seeded aggregation or cell to cell transmission. These aggregates can take various forms, including amyloid plaques, neurofibrillary tangles, Lewy bodies, and others, contingent upon the specific disease. Inflammation and oxidative stress are major mechanisms that can trigger the formation and accumulation of misfolded protein aggregates in neurodegenerative diseases. AGEs by engagement with their receptor RAGE activate a vicious cycle, where inflammation triggers oxidative stress, which in turn exacerbates inflammation and contributes to eventual neuronal dysfunction and death. Recent studies have linked diabetes to a higher risk of developing neurodegenerative diseases, specifically Alzheimer's disease, and referred to it as "type 3 diabetes" due to shared elements such as insulin resistance and brain glucose metabolism. A significant knowledge gap exists in understanding the interaction between type 2 diabetes and neurodegeneration. This study aimed to bridge that gap.

We investigated AGEs accumulation, RAGE expression, inflammation, and oxidative stress in the cortex, hippocampus, and cerebellum of streptozotocin (STZ-) induced chronic diabetic male Wistar rats and their age-matched non-diabetic control rats. The diabetic rats showed marked increases in plasma glucose levels, and oxidative stress [(increased malondialdehyde (MDA) level and decreased catalase (CAT), and superoxide dismutase (SOD) as well as reduced glutathione (GSH)] as compared to non-diabetic rats. Moreover, nuclear factor kappa B (NF-κB) p65 unit, interleukin 6 (IL-6), and C-reactive protein (CRP) were elevated while nerve growth factor-beta (βNGF) and insulin receptor substrate-1 (IRS1) were downregulated in diabetic rats. Overall, results reveal that prolonged diabetes and AGEs accumulation exacerbate oxidative stress, inflammation, RAGE expression, and insulin resistance in the brain, contributing to diabetes-associated neurodegeneration. These findings offer promising avenues for devising potential therapies targeting diabetes-related pathways in neurodegeneration.

(P-034) Complement C3 enables nephritogenic activity of galactose-deficient-IgA1-containing immune complexes in IgA nephropathy

Stacy Hall[1], Graham Gurganus[1], Zhi-Qiang Huang[1], Barbora Knoppova[1], Shihong Qiu[1], R. Glenn King[1], Shigeaki Nakazawa[1], Nicolas Maillard[2], Zina Moldoveanu[1], Dana V. Rizk[1], Bruce A. Julian[1], Matthew B. Renfrow[1], Todd J. Green[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 pathogenic circulating immune complexes (IC) consist of IgA1 with some *O*-glycans deficient in galactose (galactose-deficient IgA1; Gd-IgA1) bound by IgG autoantibodies. Additional serum components, such as complement proteins, can be associated with these IC. Some of these Gd-IgA1-containing IC enter glomeruli, activate mesangial cells, and induce kidney injury. Most patients progress to kidney failure in their lifetime, requiring kidney replacement therapy. However, IgAN recurs in most transplanted patients. Thus, it is critical to determine the precise mechanisms of disease pathogenesis and identify possible targets for development of future therapeutic approaches. In this study, we assessed the role of complement C3 in the pathogenicity of Gd-IgA1-IgG IC.

IC from native or IgA1-depleted sera of patients with IgAN were isolated by size-exclusion chromatography. Engineered IC were formed by incubating recombinant polymeric Gd-IgA1 and recombinant IgG autoantibodies specific for Gd-IgA1 in serum that was C3-depleted or C3-repleted. These engineered IC were then isolated by size-exclusion chromatography. Biological activity of the native IC from IgAN patients or engineered IC was determined based on their capacity to induce cellular proliferation of cultured primary human mesangial cells. The amount of IgA, IgG, Gd-IgA1, and of complexed IgA with IgG and IgA with C3 were determined by ELISA. SDS-PAGE under non-reducing conditions followed by immunoblotting was used to determine the covalent association of C3 with IgA or IgG. Reducing conditions were used to determine C3 processing, i.e., to detect alpha chain and/or its fragments indicative of C3, C3b, and iC3b.

IC > 700 kDa isolated from sera of IgAN patients increased cellular proliferation of quiescent mesangial cells by 2-4-fold. These IC contained IgA, IgG, and C3; C3 was covalently associated with IgA and IgG. C3 molecular forms included C3, C3b, and iC3b. Removal of IgA1 from sera removed these stimulatory IC; the resultant preparations were devoid of IgA, IgG, and C3. To confirm the role of C3, we used engineered IC formed in C3-depleted or C3-repleted serum. C3 was required for the formation of large-molecular-mass IC that stimulated cultured mesangial cells to proliferate. Moreover, SDS-PAGE immunoblotting showed that IgA and IgG formed covalent complexes with C3 in C3-repleted serum; these engineered IC contained C3, C3b, and iC3b.

In summary, complement C3 is covalently associated with Gd-IgA1-containing IC in patients with IgAN. Using engineered IC in C3-depleted or C3-repleted serum provided experimental evidence that C3 is required for a full nephritogenic capacity of Gd-IgA1-containing IC. Future experiments will determine the composition of IC of different molecular mass and/or activity.

(P-035) Therapeutic efficacy of Compound-X in vivo: A Focus on antiglycation, antioxidant, anti-Inflammatory and insulinotropic activities

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,*

Type 2 diabetes mellitus (T2DM) is a rapidly increasing global metabolic disorder, posing a significant challenge to the health and well-being worldwide. It is characterized by insulin resistance and beta-cell dysfunction. Regardless of the diabetes stage, preservation and recovery of functional pancreatic beta cells stands as the biggest challenge in the treatment of diabetes. The complications associated with diabetes mellitus significantly increase morbidity, mortality, and the risk of other diseases. Advanced glycation end products (AGEs), accumulated due to hyperglycemia, play a dual role as both cause and effect in diabetes mellitus. AGEs interact with the receptor for AGEs (RAGE), a multi-ligand well-characterized cell surface pattern recognition receptor expressed ubiquitously, particularly on beta cells. AGEs binding to RAGE can create a positive feedback loop by increasing RAGE expression on cell surfaces. This signaling cascade contributes significantly to the dysfunction and apoptosis (cell death) of pancreatic beta cells and are critical in the pathogenesis of diabetes mellitus. The oxidative stress implicated in the progression of pancreatic beta-cell demise 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. Although various oral antidiabetic medications are available with distinct mechanisms of action, none effectively counteract the detrimental accumulation of AGEs or RAGE expression within pancreatic beta cells. Understanding these challenges underscores the critical need for deeper insights into T2DM's pathophysiology and therapeutic strategies.

We evaluated the effect of Compound-X on the pancreas of streptozotocin (STZ-) induced chronic four months diabetic rats and compared the effects with Metformin and Glibenclamide as positive control. Compound-X administration for 40 days to

diabetic rats significantly detoxified the AGEs level, declined RAGE expression, normalized oxidative stress biomarkers, and CRP. Despite the fact that pancreatic beta cells regeneration is controversial, the literature provides evidence that pancreatic beta cells can regenerate from pre-existing beta cells provided with extra pancreatic stimulators. A significant increase in insulin-positive beta-cells and improved pancreatic islets integrity was observed in the diabetic Compound-X treated group as compared to diabetic control. The novelty of our research lies in Compound-X's regenerative effect on pancreatic beta-cell mass, mediated through its antioxidant properties, AGEs detoxification capabilities, and regulation of transcription factors crucial for beta-cell survival and stress resistance. The potential of Compound-X to revive and regenerate pancreatic functional beta cells mass can prove to be the breakthrough therapy for beta cells regeneration in type 2 diabetes mellitus.

(P-036) The N-glycosylation and dolichol biosynthesis defects in Lec5 and Lec9 CHO cells are caused by absence of the DHRSX gene

Matthew P. Wilson[1], Takfarinas Kentache[2], Charlotte R. Althoff[1,3], Francesco Caligiore[2], Erika Souche[4], Céline Schulz[3], Julie Graff[2], Eline Pieters[1], Pamela Stanley[5], Joseph N. Contessa[6,7], Emile Van Schaftingen[2], Gert Matthijs[1], François Foulquier[3], Guido T. Bommer[2]

[1]Laboratory for Molecular Diagnosis, Center for Human Genetics, KU Leuven, Leuven 3000, Belgium, [2]Metabolic Research Group, de Duve Institute & WELRI, Université Catholique de Louvain, 1200 Brussels, Belgium, [3]Univ. Lille, CNRS, UMR 8576 – UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F- 59000 Lille, France, [4]Laboratory for Cytogenetics and Genome Research, Department of Human Genetics, KU Leuven, B-3000 Leuven, Belgium, [5]Department of Cell Biology, Albert Einstein College of Medicine, New York, NY, USA, [6]Department of Therapeutic Radiology, Yale School of Medicine, New Haven, CT 06511, USA, [7]Department of Pharmacology, Yale School of Medicine, New Haven, CT 06511, USA,

Glycosylation-deficient Chinese Hamster Ovary (CHO) cell lines have been instrumental in the discovery of N-glycosylation machinery. More than 40 different complementation groups have been characterized in this way, mainly by selection for resistance to toxic lectins which bind specific glycans. These cells are usually referred to as CHO 'Lec' cell lines. In many of these cells, causal mutations in glycosylation pathway genes have been identified. However, the molecular causes of the glycosylation defects in the CHO Lec5 and Lec9 mutants have been elusive, despite the identification of defective dolichol formation from polyprenol in both cell lines. We recently found that, in humans, dolichol synthesis from polyprenol occurs in three steps consisting of the conversion of polyprenol to polyprenal by DHRSX, the reduction of polyprenal to dolichal by SRD5A3 and the reduction of dolichal to dolichol, again by DHRSX. This led us to investigate defective dolichol synthesis in CHO Lec5 and Lec9 cells. As previously described, both cell lines showed increased levels of polyprenol and its derivatives, concomitant with decreased levels of dolichol and derivatives. However, we found no change in polyprenal levels, suggesting DHRSX deficiency. Accordingly, N-glycan synthesis and changes in polyisoprenoid levels could be corrected by complementation with human DHRSX but not SRD5A3. Furthermore, the typical polyprenol dehydrogenase and dolichal reductase activities of DHRSX were absent in membrane preparations derived from CHO Lec5 and Lec9 cells, while the reduction of polyprenal to dolichal, catalyzed by SRD5A3, was unaffected. Long-read whole genome sequencing of CHO Lec5 and Lec9 cells showed that the genomic region containing *DHRSX* was absent as part of a deletion of at least 19Mb, including part or all of two unplaced contigs (NW_023276919.1 and NW_023276808.1). No mutations were identified in the ORF of *SRD5A3*. Using CHO Pro-5 cells, we established the sequence of Chinese hamster DHRSX and validated that this protein has similar kinetic properties to the human enzyme. Our work therefore identifies the basis of the N-glycosylation and dolichol synthesis defects in CHO Lec5 and Lec9 cells.

(P-037) Recent advances in cell biology of tissue-specific galectins

Alexander V. Timoshenko[1]

[1]Department of Biology, The University of Western Ontario, London, ON, Canada,

Human galectins are encoded by 15 different genes (LGALS1-LGALS4, LGALS7, LGALS7B, LGALS8, LGALS9, LGALS9B, LGALS9C, CLC/LGALS10, LGALSL12-LGALS14, LGALS16) and this network is supplemented with at least 11 additional galectin-related protein-coding and noncoding genes. Some galectins are commonly detected in many tissues while others are exclusive to specific cells or tissues, e.g. *LGALS12* in adipocytes, myeloid cells, and breast or *LGALS16* in the placenta and brain. The expression of common galectins is usually upregulated in cancer cells and tissues, which is not always a case for tissue-specific galectins. As such, clinical trials with galectin inhibitors for targeted cancer therapeutics are not conclusive yet and further insights are required to address these challenges. In this context, the association of galectins with processes of cellular differentiation is often overlooked as an important option of the concept of differentiation therapy of cancer. My laboratory

elaborates the hypothesis that tissue-specific galectins may serve as tumor suppressors resulted from their upregulation upon cell differentiation and their downregulation in cancer. We have used different cell culture models of cell differentiation to study the expression of highly tissue-specific *LGALS12* and *LGALS16* genes in human cell lines representing breast cancer, leukemia, choriocarcinoma, and glioblastoma. Our findings revealed that retinoic acid-induced neutrophilic differentiation of HL-60 cells and epithelial-like differentiation of MDA-MB-231 cells were associated with upregulation of *LGALS12*. Moreover, we demonstrated that galectin-12, a protein with a high hydrophobic index, was implicated in neutrophilic polarization of HL-60 cells and its secretion interfered with the formation of lipid droplets in cells [1]. Our findings provided the first evidence that *LGALS16*, a novel and largely unexplored member of galectin family, was not only significantly upregulated in JEG-3, BeWo, and U87 cells (originated from placenta and brain tissues) through cAMP-mediated signaling pathways but was required for trophoblastic differentiation of JEG-3 cells [2,3]. Ongoing research includes further characterization of molecular mechanisms underlying the potential role of tissue-specific galectins in the differentiation programs of relevant cell lineages and their utility as tumor suppressors and prognostic biomarkers.

References

Tazhitdinova R et al. *J Cell Physiol*. 2024:**239**(6):e31288. doi: https://doi.org/10.1002/jcp.31288.
Kaminker JD, Timoshenko AV. *Biomolecules*. 2021:***11***(12):1909. doi: https://doi.org/10.3390/biom11121909.
Kaminker JD et al. *Cell Biol Int*. 2024:***48***(3):378–385. doi: https://doi.org/10.1002/cbin.12128.

(P-038) Metabolically volatile malto-oligoglucans are associated with neurodegenerative polyglucosan body diseases

Silvia Nitschke[1], Alina P. Montalbano[1], Megan E. Whiting[1], Brandon H. Smith[1], Neije Mukherjee-Roy[1], Charlotte R. Marchioni[1], Mitchell A. Sullivan[2], Xiaochu Zhao[3], Peixiang Wang[3], Howard Mount[4], Mayank Verma[1], Berge A. Minassian[1], Felix Nitschke[1]

[1]*University of Texas Southwestern Medical Center, Dallas, USA,* [2]*University of Queensland, Brisbane, Australia,* [3]*The Hospital for Sick Children Research Institute, Toronto, Canada,* [4]*University of Toronto, Canada,*

Brain polyglucosan bodies (PBs) cause fatal neurodegenerative diseases including Lafora (LD) and adult polyglucosan body (ABPD) diseases, both without treatments, biomarkers, or well-understood pathogenesis. Polyglucosans are glycogen molecules with overlong chains and in LD excessive phosphorylation. Previous results in muscle of two LD mouse models (*Epm2a*$^{-/-}$, *Nhlrc1*$^{-/-}$) and one APBD mouse model (*Gbe1*$^{Y329S/Y329S}$) revealed that soluble glycogen (i.e. α-glucan) levels are WT-like, while accumulated insoluble glycogen is responsible for the observed elevated total glycogen levels in the mutants. We now analyzed brain glycogen in these mouse models at 7 months (APBD) and 10 months (LD), respectively, and used focussed microwave (FM) brain fixation for maximal preservation of the volatile brain glycogen. In contrast to previous findings in muscle, all three mutants contained significantly increased levels of soluble brain α-glucan material compared to WT. However, in age-matched mutant brains soluble α-glucan was not elevated when brains were fixed by the slower method of cryopreservation (CP). This indicates that soluble α-glucan pools in the three mutants are more metabolically volatile and degradation-prone, leading to a faster post-mortem depletion than in the respective WTs. In all three mutants total α-glucan contents determined as amyloglucosidase-released glucose after extraction in a buffer with only mechanical force were consistently higher than when total glycogen was measured after extraction in heated 30% KOH with subsequent repeated ethanol precipitations. We hypothesized that the glucan moieties lost during precipitation constitute the metabolically volatile excess soluble α-glucan in the three mutants. We subsequently analyzed the soluble α-glucans of FM-fixed mutant and WT brains after ultra-filtration, separating macromolecular glycogen and lower molecular weight α-glucan by a 30 kDa size cut-off. In the WTs virtually all soluble α-glucans were macromolecular (> 30 kDa), while the LD models had comparable and APBD mice even decreased levels compared to WT. This confirmed that the excess soluble α-glucans found in the mutants after FM fixation are in fact low molecular weight α-glucans, namely malto-oligoglucans (MOGs). We determined α-glucans in FM-fixed brains of 5-week-old LD mice and found no evidence for MOG at this age yet. MOG contents in LD mice correlated particularly well with mRNA levels of the neuroinflammatory markers *Lcn2*, *Cxcl10*, and *Ccl5* with correlation coefficients largely > 0.6, indicating a connection between neuroinflammation and MOG production. Our work identified MOGs as a metabolically volatile metabolite pool that is associated with neuroinflammatory processes in three PB disease models. MOGs are metabolized much faster than brain glycogen, which may suggest a role in acute hypoxia/hypoglycemia protection, a role that had previously been ascribed to brain glycogen.

(P-039) Desialylation of insulin-like growth factor 1 receptor sensitizes ovarian cancer cells to IGF1R inhibitor

Ayon A. Hassan[1,2], Qiushi Chen[3], Xuechen Li[3], Alice S.T. Wong[1,2]

[1]School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, [2]Laboratory for Synthetic Chemistry and Chemical Biology, Hong Kong Science and Technology Park, Hong Kong, [3]Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong,

Despite recent advances in cancer treatment, ovarian cancer remains the most lethal gynecological malignancy with limited options for treatment of advanced disease. The insulin-like growth factor 1 receptor (IGF1R) is a receptor tyrosine kinase that is hyperactive in several cancers, including ovarian cancer, and plays a crucial role in disease progression. To date, many anti-IGF1R therapeutics have entered clinical trial, but none have achieved sufficient efficacy to receive approval. In this study we investigated the function of posttranslational modification, particularly terminal glycan modifications (sialylation and fucosylation), on IGF1R inhibition in ovarian cancer. We found that treatment with sialyltransferase inhibitor (STI), but not fucosyltransferase inhibitor, sensitizes ovarian cancer cells to IGF1R inhibitor AG1024. Desialylated IGF1R was more prone to deactivation by AG1024 and resulted in decreased signal transduction by IGF1R substrates IRS and SHC. Using a combination of glycosidases and lectins, we found that IGF1R primarily carries sialic acids in α-2,6 linkage on N-glycans. With the help of mass spectrometry, we have identified the amino acid residues and glycan structure containing sialic acid. In a xenograft model of ovarian cancer, treatment with STI sensitized ovarian cancer cells to IGF1R inhibition, resulting in decreased metastatic spread onto mesenteric membrane. Taken together, our results demonstrate that sialylated N-glycans on IGF1R controls its effective inhibition and that targeting it can potentially sensitize it to anti-IGF1R therapeutics in the clinic. [Supported by RGC SRFS2223-7S05]

(P-040) Abnormal Trafficking and Processing of Multiple Matrix Metalloproteinases Drives Cartilage Defects in PMM2-CDG

Chia-Lun Wu[1], Richard Steet[1], Heather Flanagan-Steet[1]

[1]JC Self Research Institute, Greenwood Genetic Center, Greenwood, SC 29646,

Congenital disorders of glycosylation (CDGs) are a group of rare genetic diseases caused by defects in protein and lipid glycosylation. One of the most common CDG, PMM2-CDG, is caused by variants in phosphomannomutase 2 (*PMM2*). PMM2 deficiency limits production of GDP-mannose, impacting the generation of N- (and possibly O-) linked glycans. The lack of information linking misglycosylated proteins with clinical phenotypes has impeded our understanding of the mechanisms driving pathology and hampered therapy development. Using a zebrafish model of PMM2-CDG, we previously identified protein proconvertases and matrix metalloproteinases (Mmps) as key drivers of abnormal cartilage development. By combining biochemical analyses with developmental phenotyping, we showed abnormal processing of a subset of proprotein convertases and Mmps disrupts their activity. This in turn inhibits the processing of cell adhesion molecules, like N-cadherin. More recent studies show increased abundance of the mature membrane-bound form of MT1-Mmp (the activator of Mmp2) correlates with the reduction in Mmp2 activation in *pmm2* mutants. Using a zebrafish reporter line (EMMA, epitope-mediated MMP activation) that expresses a multi-tagged form of Mmp2, we found its altered activation corresponds with a reduction in secretion. Confocal analyses show pro-Mmp2 is trapped in the secretory pathway of *pmm2* mutant chondrocytes, predominantly persisting in the Golgi not the ER. While this is consistent with the reduction in Mmp2 activation, it is currently unclear why its trafficking is disrupted. We hypothesize that misglycosylation of one or both of these enzymes (Mmp2 or its activator MT1-Mmp) functionally impairs their interaction, hindering normal processing of pro-Mmp2. Data from proximity ligation assays suggest Mmp2 and MT1-Mmp normally interact in the Golgi and subsequently traffic together to the cell surface – where Mmp2 activation occurs. EndoH and PNGase treatment of immunoprecipitated protein indicate MT1-Mmp, predicted to bear a single N-glycan, does carry an N-glycan in both wild type and mutant animals. Studies to assess Mmp2 glycosylation are underway. While it is still unclear if either protein is misglycosylated in *pmm2* mutants, treatment with epalrestat (an aldose reductase inhibitor shown to modulate metabolic profiles and glycosylation in PMM2-CDG patient cells) improves processing of MT1-Mmp and Mmp2. Defining how misglycosylation relates to impaired protease activation is expected to inform mechanisms driving altered tissue development in the context of CDG, and also highlight how glycans regulate normal protease function.

(P-041) MUC1-Derived Synthetic Glycopeptide Combinatorial Libraries Containing sTn antigen

Ivet Boneva[1], Ramya Ayyalasomayajula[1], Andrew Whyte Jr[1], Maré Cudic[1]

[1]*Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, United States,*

Mucin 1 (MUC1) is a highly glycosylated transmembrane protein, which has emerged as a major target for the development of cancer therapeutics and vaccines. Approaches to MUC1-based immunotherapy rely on exploiting the overexpression and aberrant glycosylation of MUC1 in multiple cancer types. The isolation of well-defined tumor associated MUC1 from natural sources has proven to be difficult due to the natural epitope heterogeneity found on the surface of cancer cells. Our approach involves synthesis of a diverse library of aberrantly glycosylated tumor associated 20-mer MUC1 peptides, harboring five possible glycosylation sites (Thr^4, Ser^5, Thr^9, Ser^{15}, and Thr^{16}), as a tool to model the diversity in the type and positions of *O*-glycans on MUC1. A key step in the design and development of the glycopeptide combinatorial libraries is determination of the isokinetic ratios, that ultimately yield equal molar mixtures of the MUC1 glycopeptides. A protocol was established for the simple mixture of two components, the pentafluorophenyl esters of glycosylated Ser/Thr and a non-glycosylated Ser/Thr, and then the same approach was applied to the three component mixtures containing Tn, sialyl-Tn (sTn), or no glycan attached to the Ser/Thr side chain. Mixtures are incorporated in different ratios at randomized positions. The effect of neighboring glycosylation on isokinetic ratios was also evaluated. To increase the reaction rate, couplings were performed in the presence of 1-hydroxybenzotriazole (HOBt). The ratio of products formed was analyzed by RP-HPLC and compound identity was confirmed by MALDI-TOF. The isokinetic ratios for each of the five glycan positions were determined within the limits of experimental error. The optimal ratios for the *O*-glycosylated Tn and sTn building blocks were found to be similar. The positional scanning approach was used for the synthesis of a MUC1-derived glycopeptide library displaying native-like heterogeneous and aberrant *O*-glycan epitopes (Tn and/or sTn) as seen on the surface of cancer cells. The glycopeptide library was prepared by "tea bag" approach using standard Fmoc solid-phase peptide synthesis (SPPS). Enzyme-linked lectin assay (ELLA) was used to study epitope heterogeneity, the glycoside cluster effect, and the steric hindrance effect of neighboring glycans on binding to macrophage galactose lectin (MGL).

(P-042) High Density of MUC-type O-glycans Plays a Role in the Proteolytic Stability of APP Glycopeptides and Aβ40 Fibrillation

Nancy Vela[1], Gustavo Mundim[1], Ramya Ayyalasomayajula[1], Ivet Boneva[1], Deepika Regmi[1], Deguo Du[1], Dmitriy Minond[2], Maré Cudic[1]

[1]*Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, United States,* [2]*College of Pharmacy and Rumbaugh-Goodwin Institute for Cancer Research, Nova Southeastern University, Fort Lauderdale, FL 33314, United States,*

Alzheimer's disease (AD) is the most common neurodegenerative disorder linked to aging. Its profound effect on the economy, healthcare system, and society is projected to increase even further as the population ages. Evidence continues to support the idea that deficiencies in amyloid-precursor protein (APP) trafficking and clearance of amyloid-β (Aβ) peptides are the initiating events of AD pathogenic processes. Efforts to understand the role of proteolytic cleavage of APP by α-, β-, and γ-secretases into the toxic amyloidogenic pathway have sparked interest in the role of MUC-type *O*-glycosylation in this process. Recently, we provided insight into glycosylation-driven changes in the intrinsic properties of APP-derived glycopeptide models. We have synthesized native and Swedish-mutated ($Lys^{670}Asn/Met^{671}Leu$) APP glycopeptide models which include the Aβ-(1-23) region with extended *N*-terminal domain to incorporate the β-secretase cleavage site, displaying *O*-GalNAc moieties on Tyr^{681}, Thr^{663} and/or Ser^{667} to explore the role of glycan density near the β-secretase (BACE-1) and/or α-secretase (ADAM-10) cleavage sites on conformation and secretase activity, as well as aggregation kinetics of Aβ40. CD analysis was performed in three relevant solvent systems to evaluate the peptide environment and *O*-glycosylation-induced conformational changes driven by multiple glycans. Activity levels of BACE-1 and ADAM-10 were measured using high-performance liquid chromatography and characterized by mass spectrometry. A Thioflavin T binding assay was used to determine the effects of APP glycopeptides on Aβ40 aggregation while atomic force microscopy (AFM) imaged the morphology of these aggregates. The Swedish mutation and the diverse *O*-glycosylation sites were the key factors driving conformational changes. Furthermore, the level of β-secretase activity significantly increases for the glycopeptides containing the Swedish mutation whereas the presence of multiple glycans supports the non-amyloidogenic pathway. Lastly, the glycopeptides impact the kinetics of Aβ40 aggregation by significantly increasing the lag phase and delaying aggregation onset in the case of multivalent glycan presentation. However, the Swedish-mutated glycopeptides display a less significant effect on the delay of aggregation, and those bearing a GalNAc moiety on the Ser^{667} position leaning towards increased fibril formation, suggesting the important role of this mutation alongside site-specific glycosylation in the amyloidogenesis process.

(P-043) Unveiling the Role of ST6Gal1 Mediated α2,6 sialylation in Regulating Pan-Metabolic Shifts in Glioblastoma: Insights into GLUT3 dynamics

Joelle Saad[1], G.C. Sajina[1], Travis Nemkov[2], Kaysaw Tuy[1], Sam Gary[1], James A. Mobley[3], 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]O'Neal Comprehensive Cancer Center, Mass Spectrometry and Proteomics Shared Facility, University of Alabama at Birmingham, AL, USA,

Despite the importance of glycosylation in regulating protein folding, modulating cell-cell interaction, and modifying signal transduction, its pivotal role has been somewhat overlooked in diseases and malignancies, unlike other post translational modifications like phosphorylation. Beta-galactosidase α2,6-sialytransferase 1 (ST6Gal1) is a glycotransferase in the Golgi that adds a terminal α2,6-linked sialic acid onto the galactose of N-linked glycans (α2,6 sialylation), consequently modulating the dynamics and function of proteins destined for the cell surface and secretion. Addition of the α2,6-linked sialic acid is likely context dependent, in part, resulting from environmental pressures. The role of ST6Gal1 mediated α2,6-sialylation remains a topic of intense research in several fields including cancer and brain health. Recently, our lab challenged previous work that undermined the importance of sialylation in glioblastoma (GBM), the most frequent and highly malignant primary brain tumor in individuals over 50 years of age, and we identified increased ST6Gal1 as a critical regulator of stemness in a subpopulation of therapy resistant and metabolically plastic brain tumor initiating cells (BTICs). Alterations in BTIC metabolic profiles represent one mechanism underlying their resistance and survival in the harsh tumor microenvironment. However, the interplay between α2,6 sialylation and metabolism is understudied. A pressing need therefore exists to decipher the role of α2,6-sialylated proteins, including receptors involved in regulating GBM metabolism. To do so, we performed untargeted metabolomics on GBM patient derived xenografts with ST6Gal1 modulation. Our global metabolomic profile data shows altered metabolic pathways with the loss of ST6Gal1, namely: glycolysis, fatty acid oxidation, nitrogen metabolism, and polyamine metabolism. This is the first report linking changes in ST6Gal1 to global metabolome effects in GBM. Knowing that α2,6 sialylation by ST6Gal1 can alter conformation, clustering, and retention of glycoproteins including metabolic receptors, and to complement our metabolomic analysis, we pulled down sialylated proteins and performed a proteomics analysis to identify attractive downstream targets. One such target was glucose transporter 3 (GLUT3), the primary transporter responsible for glucose uptake in BTICs. Interestingly, GLUT3 has a single N-glycosylation site; however, the biological consequence of GLUT3 α2,6 sialylation remains unknown. Using high resolution imaging, we aim to study GLUT3 cell-surface dynamics and function with ST6Gal1 modulation. Taken together, our work will elucidate the effect of ST6Gal1-mediated α2,6 sialylation of metabolic receptors on metabolism uncovering novel GBM reprogramming pathways and potential therapeutic targets.

(P-044) Revisiting of role of Eogt, atypical O-glycan synthase, based on tissue distribution

Yuji Kondo[1], Kaori Kawai[1], Noriko Toida[1], Tetsuya Okajima[1]

[1]Nagoya University, School of Medicine,

The development and maintenance of vasculature are finely regulated by VEGF and Notch signaling in endothelial cells brought by surrounding tissues. Especially, Notch signaling is critical for arterial and venous endothelial cell specification at angioblast stage. Notch receptor protein is composed of large extracellular domain consisting of multiple epidermal growth factor (EGF)-like repeat, transmembrane domain, and intracellular domain (ICD). Importantly, Notch EGF domains undergo unique atypical O-linked glycosylation; O-fucose, O-glycose, and O-N-acetyl glucosamine (O-GlcNAc), and those glycans can modify preferences of ligand binding, cell surface protein expression, or stability. Upon ligand binding, Notch ICD is liberated by proteolysis, and translocates into nucleus to drive Notch signaling target genes. Here we report a highly restricted tissue distribution of an endoplasmic reticulum-localized O-GlcNAc transferase (hereafter, Eogt) to unique blood vessels, suggesting tissue specific extracellular O-GlcNAc modification of Notch receptor protein. To unveil role of Eogt in those cells, primary endothelial cells from floxed Eogt mice were isolated and applied to mechanical stress mimicking in vivo situation. Upon temporal control of Eogt deletion, we found that endothelial cell stability molecules are being affected, and treatment with Notch signaling inhibitor on control cells mirrors the phenotype, suggesting that Eogt is a modifier governing endothelial structural stability probably through O-GlcNAcylation of Notch receptor protein.

(P-045) Cell-surface glycosylation identifies subpopulations of B cells in IgA nephropathy with distinct signaling corresponding to the production of the main autoantigen, galactose-deficient IgA1

Colin Reily[1,2], Taylor Person[2], Dana V. Rizk[1], Jan Novak[2]

[1]*Department of Medicine, Nephrology Division, University of Alabama at Birmingham, Birmingham, AL, United States,* [2]*Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, United States,*

Patients with IgA nephropathy (IgAN) have elevated levels of circulating autoantigen, galactose-deficient IgA1 (Gd-IgA1), that is recognized by IgG autoantibodies. Some of the resultant immune complexes deposit in the glomeruli and induce kidney injury. Based on the polymeric form of Gd-IgA1 in the circulating immune complexes and synpharyngitic hematuria at disease onset/activity, we hypothesize that only a subpopulation(s) of IgA1-secreting cells is responsible for Gd-IgA1 production, and that abnormal cellular responses further enhance Gd-IgA1 levels in circulation. However, as the autoantigen is released from B cells, identification of those subpopulations is difficult. Here we report that distinct cell-surface glycosylation and cellular responses to CpG-ODN (oligodeoxynucleotides) stimulation, are associated with Gd-IgA1 production and distinct intracellular signaling.

Immortalized IgA1-producing cells derived from peripheral blood of IgAN patients and healthy controls were stimulated with CpG-ODN (5 ug/mL) for 30 min and then stained with HPA and PNA to detect cell-surface GalNAc and GalNAc-Gal glycoconjugates and anti-IgA antibody to identify IgA-positive cells. Intracellular signaling was assessed using fluorochrome-conjugated antibodies specific for pSTAT1, pSTAT3, pSTAT5, pSTAT6, p38-MAPK, pERK1/2, p65-NF-kB, with flow cytometry readout using a BD FACSymphony with spectral compensation. Cells were incubated for 48 h for analyses of IgA and Gd-IgA1 in cell-culture supernatants by ELISA.

CpG stimulation decreased the number of HPA- and PNA-positive cells ($p < 0.05$) as well as the amount (detected as median fluorescence index; MFI) ($P < 0.05$). Cells with high HPA vs. low HPA differed in baseline and CpG-induced pSTAT3 ($P < 0.02$), pSTAT5 ($P < 0.01$), and pMEK1/2 ($P < 0.01$). Furthermore, cells that exhibited Gd-IgA1 production after CpG stimulation had high HPA^+/PNA^+ ratio, p38-MAPK ($P < 0.01$), pSTAT3 ($P < 0.01$), and p65 NF-kB ($p = 0.05$).

Differential cell-surface glycosylation identified differential cellular signaling, both baseline and CpG-induced, that correlated with Gd-IgA1 production. Further characterization of these cell subpopulations will provide insight into mechanisms involved in IgAN pathobiology.

(P-046) MUC-type *O*-glycosylation Affects the Binding to Human Serum Proteins and GalNAc-Specific Lectins

Gustavo Mundim[1], Nancy Vela[1], Ivet Boneva[1], Ramya Ayyalasomayajula[1], Maré Cudic[1]

[1]*Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, United States,*

The irregular processing and clearance of the Amyloid-Beta Precursor Protein (APP), a type 1 transmembrane glycoprotein, by β- and γ-secretases leads to the release of neurotoxic amyloid-β (Aβ) peptides, a hallmark of Alzheimer's Disease (AD). A small percentage of AD is also caused by single genetic mutations that are passed down through families known as familial AD (FAD). The Swedish double mutation (Lys670Asn/Met671Leu), located near the *N*-terminus region of Aβ, results in a significant increase in the total output of Aβ40 and Aβ42 by providing a better substrate for the β-secretase enzyme. Additionally, recent studies have detailed the presence of mucin-type *O*-glycans proximal to enzymatically active sites on APP, playing a role in its processing fate. Considering that numerous endogenous proteins interacting with Aβ can modulate its amyloidogenic process, it is of crucial importance to assess the role of glycosylation in these processes. Furthermore, plasma proteins may mediate AD-linked inflammatory responses, providing an exciting lead in uncovering the underlying mechanisms of AD pathogenesis. Interestingly, a unique type of tyrosine *O*-glycosylation, found within short peptide fragments from AD patients' cerebrospinal fluid (CSF), acts as a ligand for the human macrophage galactose-type lectin (hMGL). Therefore, to gain a better insight into the role of glycosylation and Swedish mutation in AD, we synthesized APP glycopeptide models with a sequence that includes Aβ-(1-23), bearing the *O*-GalNAc moiety on Tyr^{681} and either IKTEEISEVKM or IKTEEISEVNL (NL=Swedish mutation) at the *N*-terminus to incorporate β-(BACE1) and α-secretase (ADAM10) cleavage sites located near and within the Aβ40 domain, bearing the *O*-glycosylation site at Ser^{667} and/or at Thr^{663}. The stability and/or binding of the (glyco)peptide models in human sera was determined by RP-HPLC and ELISA. To determine the effect of different glycosylation sites on the binding affinity to hMGL, an enzyme-linked lectin assay (ELLA) screen against hMGL was conducted.

(P-047) Characterization of a TPR OGT-CDG intellectual disability variant

Benedetta Attianese[1], Conor W. Mitchell[1,2], Stephanie Grünewald[3], Andrew T. Ferenbach[1], Daan M.F. van Aalten[1,2]

[1]Deprtament of Molecular Biology and Genetics, Aarhus University, Denmark, [2]Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee, UK, [3]Great Ormond Street Hospital for Children and Institute for Child Health, NIHR Biomedical Research Centre, London, UK,

O-GlcNAcylation is a dynamic post-translational modification involving the addition or removal of an O-GlcNAc moiety to serine or threonine residues. It is mediated by the enzymes OGT, responsible for its addition, and OGA, responsible for its removal. Recently, mutations in OGT were found in patients affected by Intellectual Disability (ID), a neurodevelopmental disorder characterized by impairments in intellectual and adaptive functions. These mutations gave rise to the definition of a novel type of neurodevelopmental disorder (NDD) called "Congenital Disorders of Glycosylation: OGT-CDG", whose biological mechanisms are not yet fully understood.

Here we investigate the effect of an OGT-CDG missense mutation located in the OGT's tetratricopeptide repeat (TPR) domain: Y180C. *In vitro* and *in cellulo* approaches have been used to study protein function and stability. To shed light over the impact of this mutation during early neurogenesis, a mouse embryonic stem cell line, carrying the specific mutation, has been created using CRISPR-Cas9 tecnology. Preliminary results shows no differences on OGT, OGA and global O-GlcNAc protein levels. Neuronal differentiation, together with biomolecular and functional analyses will be performed to further investigate this observation.

(P-048) Sialylation-mediated regulation of the nervous system: Genetic and cellular mechanisms uncovered by the Drosophila model

Boris Novikov[1], Pedro Monagas-Valentin[1], Horton Richard[1], Alex Choi[1], Guang Lin[2], Hugo Bellen[2], Vladislav Panin[1,†]

[1]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA, [2]Departments of Molecular and Human Genetics and Neuroscience, Baylor College of Medicine, Houston, TX, USA,

[†]Presenter

The brain is the organ with the most prominent sialylation in the human body. Recent studies have implicated defects in sialylation in several neurological diseases, underscoring the pivotal roles that sialylation plays in the nervous system. Despite its importance, the functional and regulatory mechanisms of this prominent type of glycosylation remain poorly understood. The overwhelming complexity of the nervous system and the limitations of in vivo approaches hinder research on neural sialylation in mammals.

Our project leverages the advantages of the Drosophila model, with its simplified and well-studied nervous system and a powerful arsenal of genetic tools, to investigate the neural functions of N-linked sialylation. In our previous studies, we characterized Drosophila sialyltransferase (DSiaT) and CMP-sialic acid synthetase (CSAS), two key enzymes of the sialylation pathway. Our results highlighted the significant evolutionary conservation of this pathway between Drosophila and mammals and demonstrated that sialylation in Drosophila is a highly regulated process, restricted to the nervous system, and required for neural development and neurophysiology.

Our recent research has discovered that neural sialylation is mediated by a unique bipartite pathway involving glia-neuron interactions. We found that sialylation promotes the function of voltage-gated sodium channels and protects against oxidative stress. Our current experiments continue to explore the mechanism of sialylation in the nervous system. We are developing new genetic tools to investigate the cell non-autonomous function of neural sialylation, including the potential involvement of extracellular vesicles in sialylation-mediated glia-neuron communications.

Our experiments indicated that sialylation genes exhibit mutant phenotypes similar to those of Gba1b, an orthologue of the glucocerebrosidase mutated in Gaucher disease. This suggests that sialylation defects and Gba1b deficiency converge on common pathogenic mechanisms. We will discuss our new data in light of the potential interplay between these pathways. Our results are expected to elucidate conserved mechanisms of neural regulation in both healthy and pathological conditions, providing valuable insights for biomedical and clinical research on neurodegenerative diseases.

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(P-049) Competition between *O*-GlycNAcylation and Phosphorylation of Tau Protein

Andrew Whyte Jr[1], Nancy Vela[1], Alejandra Coronel Zegarra[1], Vivian Merk[1], Maré Cudic[1]

[1]*Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, United States,*

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a loss of neural function. It is the most common form of dementia and afflicts millions of people worldwide with only a handful of FDA-approved drugs to alleviate symptoms. Amyloid-β (Aβ) plaques and intracellular tau protein neurofibrillary tangles (NFTs) are the hallmarks of AD, and early studies were primarily focusing on the inhibition of Aβ plaque formation. Recent studies have begun shifting focus to NFTs as the primary therapeutic target due to various failures of the Aβ inhibitors at the clinical trial stage. Considering that tau protein functions are typically regulated by a posttranslational modification (PTM), such as hyperphosphorylation, research efforts were focused on the understanding of how PTMs specifically regulate the transition of tau into pathogenic conformations, known as tauopathies, under pathological conditions. However, other PTMs, such as *O*-GlcNAcetylation, have been implicated in tau regulation, and may play a protective role against AD and other tauopathies as a modulator of hyperphosphorylation. In order to explore the role of *O*-GlcNAcetylation on tau aggregation, we synthesized the phosphorylated and glycosylated *C*-terminal fragment of the tau protein that includes PHF-1 epitope by using solid-phase peptide synthesis (SPPS). The conformational changes that are influenced by the presence or absence of different PTMs are determined by circular dichroism (CD) and the morphological changes will be assessed by atomic force microscopy (AFM) and transmission electron microscopy (TEM).

(P-050) A mouse model of O-GlcNAc transferase intellectual disability shows skull deformation and cognitive deficits

Florence Authier[1], Kevin-Sebastien Coquelin[1], Iria Esperón Abril[1], Daan M.F. van Aalten[1]

[1]*MBG, Aarhus University,*

O-GlcNAcylation is a protein modification that is critical for vertebrate development, catalysed by O-GlcNAc transferase (OGT) and reversed by O-GlcNAcase (OGA). Missense mutations in OGT have recently been shown to segregate with a syndromic form of intellectual disability, OGT-linked Congenital Disorder of Glycosylation (OGT-CDG). Although OGT-CDG suggests a critical role of O-GlcNAcylation in neurodevelopment and/or cognitive function, the underlying pathophysiologic mechanisms remain unknown. We have recently reported a mouse model that carry a catalytically impaired OGT-CDG variant. These mice show altered O-GlcNAcylation homeostasis in the brain. Phenotypic characterization of the mice revealed microcephaly, skull deformation and cognitive deficits including hyperactivity and reduced anxiety. This mouse model will serve as an important tool to study genotype-phenotype correlations in OGT-CDG in vivo and for the development of possible treatment avenues for this disorder.

(P-051) Drug Discovery for Sanfilippo Syndrome

Amrita Basu[1], Madison Intermann[2], Barry C. Maloney[2], Kimberly Cisneros[1], Antoinette C. Russell[3], Christopher A. Rice[3], 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 and Emerging Global Diseases, University of Georgia, Athens, GA,*

Mucopolysaccharidosis Type IIIA (MPS IIIA), also 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, 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 as novel substrate reduction therapies for MPS IIIA. We hypothesize that small molecule agents that lower expression of the key HS biosynthetic enzyme, EXT1, could reduce cellular HS levels and lysosomal accumulation in cells, thus restoring cell homeostasis. To investigate this, we established a high-throughput drug screening assay using CRISPR-engineered EXT1 reporter

cells and a library of FDA-approved drugs (2,320 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. Interestingly, several leading compounds from the screen focus on receptor tyrosine kinases (RTKs) and associated signaling pathways and have previously demonstrated the ability to cross the blood-brain barrier. The top drug candidates notably decreased EXT1 expression, intracellular HS levels, and lysosomal storage in fibroblasts derived from MPS III patients. Notably, the majority of the compounds also stimulated markers of autophagy. In summary, these studies have identified small molecule agents that target HS assembly, reduce lysosomal HS accumulation, and offer promising avenues for further drug development for MPS IIIA and similar disorders.

(P-052) The ST6GAL1 sialyltransferase is upregulated during pancreatitis and functions in this context to promote acinar cell survival and pancreatic tissue regeneration

Michael P. Marciel[1], Nikita Bhalerao[1], Susan L. Bellis[1]

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

Pancreatitis develops after injury to the pancreatic acini, resulting in the release of exocrine enzymes and subsequent tissue necrosis, edema, and acute inflammation. After the onset of acute pancreatitis, many acinar cells die, however a subset of cells dedifferentiates in a process known as acinar-to-ductal metaplasia (ADM). Cells undergoing ADM re-enter the cell cycle to repair tissue damage. Here we report that ADM and tissue regeneration are facilitated by the ST6GAL1 sialyltransferase. ST6GAL1 adds an α2-6 linked sialic acid to select *N*-glycosylated surface receptors, an event that remodels cell phenotype to impart pro-survival, progenitor-like characteristics. For example, α2-6 sialylation activates EGFR, a well-known player in promoting acinar cell survival and ADM. In fact, EGFR is the main receptor that directs the upregulation of ADM-driver genes such as the Sox9 transcription factor. In the current study, we discovered that ST6GAL1 was upregulated in human acute and chronic pancreatitis, and in experimental murine models of acute pancreatitis. ST6GAL1 expression was restricted to the ADM-like population, whereas ST6GAL1 was undetectable in the acinar cells of healthy pancreas. The ST6GAL1-positive, ADM-like cells displayed enhanced expression of Sox9 and the proliferative marker, Ki67. In contrast to cells undergoing ADM, apoptotic acinar cells lacked ST6GAL1 expression. To uncover a functional role for ST6GAL1 in pancreatitis, we generated mice with transgenic expression of ST6GAL1 in the pancreas (SC mice, Pdx1-Cre; LSL-ST6GAL1). Upon induction of acute pancreatitis, SC pancreata displayed a greater number of ADM-like cells compared to WT, and also exhibited a pronounced activation of EGFR. To study cell signaling we used organoids derived from SC mice as well as an immortalized human acinar cell line with ST6GAL1 overexpression (HPNE OE cells). Compared with controls, SC organoids and HPNE OE cells had greater activation of EGFR in response to EGF and TGFα, and this led to enhanced expression of Sox9. Additionally, SC organoids and HPNE OE cells were resistant to TNFα-induced apoptosis. Correspondingly, better survival of ST6GAL1-overexpressing cells was observed under conditions of cell stress including serum starvation and anchorage-independent growth. Finally, to interrogate ST6GAL1's role in tissue regeneration, we induced acute pancreatitis in WT and SC mice and monitored tissue healing. We found that SC mice had improved tissue regeneration at 3, 7, and 10 days following the induction of pancreatitis, as evidenced by the restoration of normal acinar morphology, decreased levels of tissue necrosis, reduced TUNEL staining, and reduced immune cell infiltration. These findings indicate that the ST6GAL1-mediated sialylation of select acinar receptors plays a protective role during pancreatitis and aids in pancreatic tissue recovery.

(P-053) Urine N-glycan Profiling for Classification of Systemic Lupus Erythematosus Disease Course Using the GlycoTyper™ Liquid Biopsy Platform

Gray Huffman[1], Grace Grimsley[1], Aaron Angerstein[2], Tamara Nowling[3], Richard Drake[2], Steve Castellino[1]

[1]*Glycobiology Solutions, Bruker Scientific Inc.,* [2]*Department of Cell and Molecular Pharmacology, Medical University of South Carolina,* [3]*Division of Rheumatology & Immunology, Medical University of South Carolina,*

Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect many different tissues and organs. Lupus nephritis (LN) is a serious complication of SLE where autoantibodies form immune complexes that are deposited in the glomeruli, causing inflammation, kidney damage, and potentially kidney failure. It is estimated that up to 60% of adults and 80% of children with SLE will develop LN at some point. Because SLE is a heterogeneous disease with a highly variable course, early diagnosis and aggressive treatment are crucial to prevent progression to end-stage renal disease. Currently, in the absence of any liquid-biopsy-based tests, LN diagnosis still relies on renal biopsy as the standard of care. Alternative approaches are urgently needed.

We used the GlycoTyper platform, based on MALDI-characterization of enzymatically released N-glycans from affinity-captured target proteins, to analyze urine samples from 114 healthy controls (HC), 115 SLE patients, and 139 LN patients. In addition, matched serum and urine samples of 50 LN patients were analyzed. For this application, the GlycoTyper platform

consisted of IgG capture antibody arrays printed on Nexterion-H-coated glass slides. The slides were modified to a custom multi-well format for the addition of patient samples. After IgG capture and washing steps, N-glycans were enzymatically released with PNGase F. The slides were then coated with matrix followed by N-glycan readout using MALDI mass spectrometry (Bruker timsTOF fleX). The robustness of the platform was addressed by a quality strategy which included system suitability tests during sample preparation, incorporation of QC arrays throughout the slide, and both positive and negative control arrays.

Preliminary data analysis of urine samples produced a multiclass random forest classifier with an area under the receiver operating characteristic curve of 0.86 for differentiating LN patients from patients with SLE but no kidney disease and HCs, with a sensitivity of 0.9 and a specificity of 0.82. This model was trained on the centered log ratios (CLRs) of the normalized intensities of 38 N-glycans, as well as on the age and sex of each patient. While the model performed well in differentiating LN patients from HC or SLE, urine-derived N-glycan profiles among HC and SLE samples showed no statistically significant differences. Subsequent analyses of matched serum and urine samples of LN and HC patients are currently ongoing to compare N-glycan profiles derived from IgG present in each biofluid.

(P-054) Protein O-mannosyltransferases collaborate with receptor protein tyrosine phosphatases to establish proper axon wiring of sensory and circadian clock neurons in *Drosophila*

Pedro Monagas-Valentin[1,†], Mustafa Ozturgut[2], Joy Jiang[1], Horton Richard[1], McKenna Song[1], Paul Hardin[2], Vlad Panin[1]

[1]*Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA,* [2]*Department of Biology, Texas A&M University, College Station, TX, USA,*

[†]Presenter

Protein O-mannosylation (POM) is an evolutionarily conserved type of glycosylation known to be necessary for neuromuscular development and physiology in animals [1]. 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. Using a combination of genetic, cell biological, and glycoproteomic approaches, we identified Receptor Protein Tyrosine Phosphatase 69D (PTP69D) as a novel functionally important substrate of POMT1/2 in *Drosophila* [2]. In our current research, we focus on another member of the Receptor Protein Tyrosine Phosphatase (RPTP) family, Leukocyte-antigen-related-like (Lar), which has conserved functions in a wide range of animals, from *Drosophila* to humans. *Drosophila* Lar (DLar) is structurally and functionally similar to PTP69D, making it a potential candidate for POMT1/2-dependent O-mannosylation. Our recent experiments revealed that *DLar* genetically interacts with *POMT1/2* in sensory axon wiring in the larval ventral nerve cord. Additionally, we found *DLar* and *POMT1/2* show functional interactions in modulating connectivity of a subtype of neurons involved in circadian clock regulation. Our current experiments focus on the analysis of cellular and behavioral phenotypes affected by these interactions. The molecular and genetic mechanisms underlying these phenotypes are potentially conserved in mammals and may shed new light on the involvement of POMT1/2 and RPTPs in human pathologies.

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References

Koff M et al. Protein O-mannosylation: one sugar, several pathways, many functions. *Glycobiology*. 2023:**33**(11):911–926.

Monagas-Valentin P et al. Protein tyrosine phosphatase 69D is a substrate of protein O-mannosyltransferases 1-2 that is required for the wiring of sensory axons in Drosophila. *J Biol Chem*. 2023:**299**(3):102890.

(P-055) Characterizing *N*-Glycosylation and Sialic Acid Content in Fetal Fibrinogen

Tana V. Palomino[1], Ana Sheridan[2,3], David C. Muddiman[1], Ashley Brown[2,3]

[1]*FTMS Laboratory for Human Health Research, Department of Chemistry, North Carolina State University,* [2]*Joint Department of Biomedical Engineering, North Carolina State University and University of North Carolina-Chapel Hill,* [3]*Comparitive Medicine Institute, North Carolina State University,*

Fibrinogen is a glycoprotein found in blood that helps form blood clots for wound healing. Neonates have a variant of fibrinogen known as fetal fibrinogen. Currently, neonates that are significantly bleeding during medical procedures are administered adult

blood products, which can result in inefficient blood clots that are unable to stop bleeding. Fetal fibrinogen has higher sialic acid content compared to adult fibrinogen, but the location and extent of sialylation is unknown. This work aims to investigate the differences in *N*-glycosylation and sialylation between fetal and adult fibrinogen. Using filter-aided *N*-linked glycan separation (FANGS), glycans were cleaved and eluted from fibrinogen. SDS-PAGE was used to separate the alpha, beta, and gamma chains, after which an in-gel enzymatic digestion was performed to retrieve glycans pertaining to each chain. Densitometry was conducted on the gel to estimate the amount of protein per gel band. Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry was used for this analysis. IR-MALDESI is a soft hybrid ionization technique that can ionize sialylated glycans without the use of chemical derivatization, making it an optimal candidate for investigating sialic acid content. With FANGS, a total of 39 glycans were detected in fetal fibrinogen, and 22 glycans were detected in adult fibrinogen. Glycans with masses less than 4 kDa were most abundant in fetal fibrinogen, and glycans within 4 kDa and 5 kDa were most abundant in adult fibrinogen. All detected glycans were under 5 kDa. Digestion of SDS-PAGE resulted in the detection of 4 sialylated glycans. Using densitometry, we found increased glycan abundance across all three chains, and increased sialic acid content in the beta chain. This supports previous findings where more B-knob interactions occur in fetal clot formation. By elucidating the sialic acid content difference in fetal and adult fibrinogen, research can focus on developing blood products suitable for neonates.

(P-056) Synthesis of MUC1 Peptide Backbone Bearing Tn and Sialyl-Tn Antigens for Structural and Functional Studies with Human Macrophage Galactose-Type Lectin (hMGL)

Maré Cudic[1], Ramya Ayyalasomayajula[1], Ivet Boneva[1], Andrew Whyte Jr[1], Donella Beckwith[1], Forrest G. FitzGerald[1], Sabine André[2], Herbert Kaltner[2]

[1]Department of Chemistry and Biochemistry, Charles E, Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, USA, [2]Department of Veterinary Sciences, Physiological Chemistry, Ludwig-Maximilians-Universität München, Lena-Christ-Str. 48, 82152, Planegg-Martinsried,

Mucin 1 (MUC1) is a heavily glycosylated transmembrane protein, altered in both expression and glycosylation pattern in human carcinomas of the epithelium. The incomplete or truncated glycan structures, often capped by sialic acid are overexpressed in premalignant and malignant tissue. Interactions between the tumor-associated carbohydrate antigens (TACAs) of MUC1 and the carbohydrate-binding proteins, lectins, often lead to the creation of a pro-tumor microenvironment favoring tumor initiation, progression, metastasis, and immune evasion. Macrophage galactose binding lectin (MGL) is a C-type lectin receptor found on antigen-presenting cells that facilitates the uptake of carbohydrate antigens for antigen presentation, modulating the immune response homeostasis, autoimmunity, and cancer. The specific aims of our research were to synthesize mono and multiple glycosylated MUC1 glycopeptide models carrying the Tn and/or sTn (sialyl Tn) antigens, that allow for the control of the complexity of the chemical space of the multivalent ligands. In addition, we synthesized Positional Scanning Synthetic Glycopeptide Combinatorial Libraries (PS-SGCLs) as a tool to model the diversity in the type and positions of *O*-glycans on MUC1 and to help us understand the multivalent carbohydrate–lectin recognition processes at the molecular level. Considering the crucial role of tumor-associated forms of MUC1 with MGL in tumor immunology, a thorough understanding of this interaction is essential for it to be exploited for cancer vaccine strategies. The thermodynamic profile of the binding interaction between the human MGL and MUC1 glycopeptide models was analyzed using isothermal titration calorimetry. The binding kinetics was determined by direct measurement of the strength of unbinding of receptor-ligand interactions by atomic force microscopy. These mechanistic studies revealed that the type of carbohydrate (Tn or sTn) and the density of glycosylation affected the thermodynamics and kinetics of binding of the synthesized MUC1 glycopeptides to trimeric hMGL. The prolonged lifetime of the lectin–glycan complex with increasing glycan valency, in addition to the observed proportional enhancement of ΔH, increased negative cooperativity, and increased entropic penalties led us to propose the "bind and jump" mechanism model for the interaction of MUC1 glycopeptides with the trimeric hMGL.

(P-057) Impact of Glycogen on Survival Disparities in Lung Adenocarcinoma

Harrison A. Clarke[1,2], Tara R. Hawkinson[1,2], Cameron J. Shedlock[1,2], Terrymar Medina[1,2], Roberto A. Ribas[1,2], Lei Wu[1,2], Xin Ma[1,8], Josephine E. Chang[3], Lyndsay E.A. Young[3], Jelena A. Juras[3], Michael D. Buoncristiani[3], Alexis N. James[3], Anna Rushin[1], Matthew Merritt[1], Annette Mestas[1], Jessica F. Lamb[3], Elena C. Manauis[3], Grant L. Austin[3], Li Chen[8], Pankaj K. Singh[1,2], Craig W. Vander Kooi[1,2], Christine F. Brainson[3], Derek B. Allison[3], Matthew S. Gentry[1,2], Ramon C. Sun[1,2]

[1]Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, [2]Center for Advanced Spatial Biomolecule Research, University of Florida, [3]Department of Molecular and Cellular Biochemistry, College of Medicine, University of Kentucky, [4]Department of Neuroscience, College of Medicine, University of Kentucky, Lexington, Kentucky, [5]Markey Cancer Center, University of Kentucky, Lexington, Kentucky, [6]Department of Molecular and Cellular

Biochemistry, College of Medicine, University of Kentucky, Lexington, Kentucky, [7]Department of Toxicology and Cancer Biology, College of Medicine, University of Kentucky, Lexington, Kentucky, [8]Department of Biostatistics College of Public Health and Health Professions and College of Medicine, University of Florida, Gainesville, FL, USA, [9]Department of Pathology and Laboratory Medicine, College of Medicine, University of Kentucky, Lexington, Kentucky,

Lung adenocarcinoma is a devastating disease with a disproportionately higher mortality in the Appalachian region of the United States compared to the rest of the country. We investigated the role of glycogen in lung adenocarcinoma and how it contributes to the disparity you see in the Appalachian community. Using spatial metabolomics, we exhibited elevation of glycogen in the lungs of Appalachian LUAD patients which is a downstream effect of a diet high in fats and carbohydrates due to lack of access to healthier alternatives. To model this, we used mice that were transgenic for the KrasG12D mutation and had knockout of the gene encoding p53. These mice were orally gavaged daily for 2 weeks using: vehicle control (H2O), high fructose corn syrup (HFCS, a carbohydrate), corn oil (a fat), or combination HFCS and corn oil. After this gavage period, lung tumorigenesis was initiated via intranasal delivery of AdenoCre virus. When we investigated the adenocarcinomas, we discovered that the mice that receiving the combination diet developed a higher number of tumors as well as larger tumors when compared to mice that received control treatment. These data support our hypothesis that the lung is sensitive to higher glycogen which is induced by a diet high in fats and carbohydrates. These findings offer a look into metabolic networks involved in lung adenocarcinoma as well as show a path for prevention strategies to fight against this disease.

(P-058) Stem Cell-derived Neuronal Models for Characterization of Peripheral Nervous System Dysfunction in Sanfilippo Syndrome

Meagan Shinn[1,2], Tripti Saini[2,3], Amrita Basu[1], Jennifer Art[2,3], Nadja Zeltner[2,3], 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 Georgia, Athens, GA 30602, [3]Center for Molecular Medicine, University of Georgia, Athens, GA 30602,

Mucopolysaccharidosis Type IIIA (MPS IIIA), also known as Sanfilippo Syndrome Type A, is a rare autosomal recessive disorder that affects one in every 70,000 children worldwide, and currently there are no approved therapies. Patients with MPS IIIA are unable to catabolize a subtype of cellular polysaccharides, known as heparan sulfate (HS), due to inherited mutations in the gene encoding for the lysosomal enzyme, *N*-sulfoglucosamine sulfohydrolase (SGSH). Deficiency in this enzyme leads 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. To date, MPS IIIA neuropathologic and therapeutic studies have focused predominantly on changes in the central nervous system, especially in the brain, but little is known about the disease pathology in the peripheral nervous system (PNS). Intriguingly, both MPS IIIA patients and mouse models display symptoms of degeneration of the sensory and autonomic nervous system, including retinopathy, bowel issues, and cardiomyopathy. Yet, the molecular mechanisms triggering the underlying peripheral neuropathy are virtually unknown. The main goal of this project is to develop novel disease models to identify therapeutic targets to treat PNS issues in MPS IIIA patients. To do this, we utilized patient-derived induced pluripotent stem cells (iPSCs) to generate peripheral neural stem cell models to recapitulate PNS phenotypes and dysfunction found in MPS IIIA patients. iPSCs from MPS IIIA patients with an intermediate and severe clinical phenotype, respectively, were differentiated into neural crest cells and peripheral sensory neurons and were evaluated for disease pathology, cell morphology, and neural electrophysiology. MPS IIIA peripheral sensory neurons displayed substantial lysosomal accumulation of HS, as measured via mass spectrometry and fluorescence microscopy methods. Overall, this work will lead to the development of novel cellular models to improve our knowledge about the molecular underpinnings of PNS dysfunction in MPS IIIA and will promote the discovery of new therapies by addressing the underlying cause of the disorder, lysosomal accumulation of heparan sulfate.

(P-059) Natural Marine-Derived Fucosylated and Sulfated Glycans Inhibit Notch Activation in Ovarian Cancer Cells

Catherine Scarrow[1], Tyler Person[1], Marwa Farrag[2], Karen L. Abbott[1,†], Vitor Pomin[2,†]

[1]Arkansas Colleges of Health Education, Oncology Research Center, Department of Biochemistry and Cell Biology, Fort Smith, AR USA, [2]University of Mississippi, Department of BioMolecular Sciences, University, MS USA,

[†]These principal investigators supervised the research equally

Ovarian cancer continues to have unacceptably high mortality rates. The identification of methods to intercept disease progression and recurrence are needed. Glycosylated compounds are very safe and stable as drugs, and their medical applications in blocking clotting as well as viral and bacterial pathogenesis have been successful. Prior research indicates that glycan changes, bisecting N-linked and O-fucose, that occur on glycoproteins in ovarian cancer play roles in promoting the expansion of the cancer stem cells or tumor initiating cells. The Notch signaling pathway plays a prominent role in tumorigenesis and is a high priority target for the development of new therapeutics that can block cancer stem cells. In this pilot study we show that the fucosylation and the sulfation groups on certain natural marine-derived glycans are key structural requirements for the inhibition of Notch activation. We measure the binding affinities of these natural glycans with key glycosaminoglycan-binding proteins of ovarian cancer cells such as Wnt using surface plasmon resonance. Using reporter assays for Notch, Wnt, and Hh we determine the efficacy of these natural glycans for single and multiple pathway inhibition. Finally, we demonstrate that the Notch inhibition mediated by natural marine-derived glycans works in synergy with suppression of Notch glycosylation in Radical Fringe knockout cells.

(P-060) Coley's Toxins contained Polysaccharides that were Tumor Hemorrhagic Therapeutics, 1868-2024

R.A. Laine[1,*], H.W. Lopez[2], Hiromu Takematsu[3]

[1]*Louisiana State Univ., Baton Rouge, LA, USA,* [2]*Murigenics, Inc., 941 Railroad Ave, Vallejo, CA, USA,* [3]*Fujita Health University, Toyoake, Aiichi, Japan,*

*Corresponding author: rogerlaine@gmail.com

Busch, 1866, 1868 reported nosocomial Erysipelas caused sarcoma tumor regression. Erysipelas etiologic agent, *Streptococcus erysipelatos,* (now *S. pyogenes*) Fehleisen (1880). Live cultures in cancer patients, ***erysipelas*** led to tumor regression. Spronck (1891) injected heat treated cultures into humans and dogs with cancers, tumor shrinkage. Coley (1891, 1910) independently induced ***erysipelas*** injecting live *S. pyogenes* in cancer patients if contracted ***erysipelas*** had tumor regression. Coley eventually developed mixed bacteria-filtered, heat treated *S. erysipelatos* and *S. marsescens* culture media "Coley's Toxin" (CT) for cancer. Heat stability suggests polysaccharides were the active principle (API). Parke-Davis produced CT for 20 years with broadly variable potency. Literature reviews by Novotny 1985 and Nauts 1990 showed 50% 5 year survival times with CT, 897 patients, 18 different cancers. Hartwell and Shear, 1943, isolated a *Serratia* polysaccharide inducing tumor-specific hemorrhage in mice, 1500× more potent than Parke-Davis' CT. Algire, 1947, showed Shears' fraction caused tumor-specific capillary damage in mice. *Repeating Shear's work we isolated a 250,000da polysaccharide PS1 by SEC which caused tumor hemorrhage in a mouse model.* Hellerqvist, at Vanderbilt, 1980's isolated CM101 (GBS toxin), a 270kDa glycan from *Streptococcus agalactica*. CM101 caused lung capillary damage, 30–50% fatality in human newborns Early Onset Disease. Hellerqvist hypothesized tumors also generate hypoxic capillaries, and CM101 caused tumor specific hemorrhage in mouse Madison lung cell tumors. This led to Phase I clinical trials with 33% effectivity in stage 4 patients. (DeVore, et al. 1997). *GBS toxin receptor Sialin (SLC17A5) was proposed by Fu, et al. in 2002.* Complement activation followed by an inflammatory cytokine cascade caused neutrophil capillary destruction specific to tumors. Voelz, 2010, reported nosocomial Serratia in a neonate facility. In the first 5 days 9 neonates had respiratory distress, febrility, 50% fatality similar to the early onset disease GBS effects on newborns. Capillary endothelium lectin receptors for CM101 and PS1 may be identical. *We propose that the API's of Coley's Toxin are polysaccharides CM101 and PS1.*

(P-061) Dissecting Neurodevelopmental Impacts of Mutations in the Catalytic Domain of OGT

Shagana T. Balasubramaniam[1], Sergio Costa Eduardo Almeida[1], Daan Van Aalten[1]

[1]*Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark,*

Intellectual disability (ID) affects approximately 1–3% of the global population and can result from various genetic causes. Recent research has linked mutations in the *ogt* gene to a specific form of X-linked intellectual disability known as Congenital Disorder of Glycosylation (OGT-CDG). O-linked N-acetylglucosamine (GlcNAc) transferase (OGT), an enzyme involved in O-GlcNAcylation, modifies numerous proteins and is crucial for cellular functions. OGT-CDG presents with a range of symptoms, including cognitive impairment, developmental delays, sensory deficits, and distinctive facial features. However, the mechanisms through which these genetic mutations manifest as such symptoms are not yet fully understood.

In this study, we utilize mouse models with patient-specific *ogt* mutations to investigate the effects of these mutations on brain development and function. Notably, these mice exhibit microcephaly, which provides a basis for examining cortical development and neuron density. Preliminary analyses reveal no significant changes in neuron density but suggest alterations in neuronal

morphology, specifically in the size of NeuN-stained nuclei. Additionally, we explore the impact of these mutations on microglial function, which is essential for synaptic pruning and overall brain development. Early findings indicate a reduction in IL-4 levels in the cerebellum of the affected mice, a factor critical for microglial activity. This reduction may contribute to impaired microglial function and disrupted brain development.

Further research involves comprehensive analyses of brain tissue, including detailed measurements of neuron morphology, microglial responses, and gene expression profiling. The aim is to deepen our understanding of how *ogt* mutations influence brain structure and function, potentially leading to novel insights and therapeutic strategies for OGT-CDG and related disorders.

(P-062) Determining the Substrate Specificity of the M3 Glycan Biosynthetic Enzymes

Terrell Carter[1], David Steen[1], Jeffery Fairley[1], Erin Suh[1], Robert Bridger[1], Linda Zhao[1], Jeremy Praissman[1], Osman Sheikh[1], Digant Chapla[1], Kelley Moremen[1], Lance Wells[1]

[1]*CCRC, BMB Department, UGA,*

Dystroglycanopathy is a common subtype of congenital muscular dystrophy (CMD) that presents with progressive muscle weakness and can involve severe neurological complications. Dystroglycanopathy is so named because it results from the failure of extracellular matrix components, such as laminin, to bind properly glycosylated alpha-dystroglycan (α-DG). This binding is not a protein-protein interaction but instead is a specific protein-glycan interaction. Dystroglycanopathy results from mutations in the genes encoding the glycosyltransferases that build the specific glycan structure referred to commonly as the functional M3 (fM3) glycan. Biosynthesis of the fM3 glycan requires the activity of 11 enzymes and appears to only be present on one protein, α-DG, at 3 sites. We hypothesize that localization and specificity of the M3 pathway specific enzymes ensure that only α-DG is modified with the fM3 glycan. Utilizing synthetic peptides, recombinant enzymes, and sugar nucleotides, we are building the entire fM3 glycan in vitro and and examining the specificity of the enzymes at each step. Specificity appears to be regulated by both enzyme localization, especially for early step enzymes in the endoplasmic reticulum and highly specific substrate specificity for many of the Golgi enzymes. Completion of this work will provide a better understanding of how the fM3 glycan pathway, which when deficient lead to congenital muscular dystrophy, is highly restricted so as to not modify protein sites or glycan structures other than those found on specific residues of α-DG.

(P-063) The role of PTMs in AD pathology

Maria Vezzi[1], Nancy Vela[1], Gustavo Mundim[1], Ramya Ayyalasomayajula[1], Ivet Boneva[1], Dmitriy Minond[2], Maré Cudic[1]

[1]*Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431, United States,* [2]*College of Pharmacy and Rumbaugh-Goodwin Institute for Cancer Research, Nova Southeastern University, Fort Lauderdale, FL 33314, United States,*

Protein post-translational modifications (PTMs) are an essential mechanism of eukaryotic cells to diversify protein function beyond what the genome dictates. One of the most common ones is glycosylation and phosphorylation. They often occur in clusters, thus greatly enhancing the number of chemically distinct structures in the cell. This led to the hypothesis that cells use these natural combinatorial libraries of PTMs to diversify and regulate protein functions. Recent evidence indicates that glycosylation and phosphorylation are implicated in Alzheimer's disease (AD) pathology. The identification and the effect of these modifications on the amyloid-β precursor protein (APP) function and its subsequent role in AD pathogenesis remains a rather unexplored area in AD. In this study, we took advantage of synthetic chemical strategies developed in our group to prepare APP peptide models with the site-specific glycan and/or phosphate group attachment to examine the consequences of these PTMs on the secondary structure of APP model peptides and proteolytic processing by α- and β-secretases. The mucin-type *O*-glycosylation site on the phenolic hydroxyl group of the tyrosine (Tyr681) in the proximity of α-secretase and the phosphorylation site at the Ser679 was explored. Circular dichroism (CD) was used to analyze the conformation of peptides and to evaluate peptide environment-induced conformational changes in water, sodium phosphate buffer, and in the presence of trifluoroethanol (TFE). The assessment of the *O*-GalNAcylation and/or phosphorylation on the proteolytic susceptibility of synthesized glycosylated phosphopeptide models toward α- and β-secretase was performed by HPLC. We have shown that PTMs can modulate processing, either to expedite the protein cleavage or to slow down and stabilize the protein, thus allowing to fine-tune molecular interactions leading to specific biological outcomes.

(P-064) Biochemical Characterization of Causal X-Linked Intellectual Disability Variants of the O-GlcNAc Transferase Catalytic Domain to Better Understand OGT-CDG

Johnathan Mayfield[1], Laura Holden[1], Naomi Hitefield[1], Lance Wells[1]

[1]*From the Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, 30605, United States of America,*

O-GlcNAc Transferase (OGT) is the sole enzyme responsible for the O-GlcNAc modification onto thousands of nucleocytoplasmic proteins, and mutations in this X-chromosome gene 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 by our lab and others. While no common 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 data suggesting 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 glycosyltransferases, 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. It has been previously suggested that glucosamine supplementation may be a therapeutic option for patients as it increases available UDP-GlcNAc, and if the novel catalytic domain variants are Km mutants, supplementation should alleviate the defects seen in glycosyltransferase activity. We have shown that all three catalytic domain variants are responsive to glucosamine supplementation, and the variants T570A and A952V reach wildtype levels of O-GlcNAcylation upon glucosamine supplementation. Y835C does not reach wildtype levels with supplementation likely due to a stability defect as evidenced by low levels of expression. These three novel variants all likely result in hypoglycosylation of key substrates as the result of an altered Km or altered stability. We have purified full-length mammalian-expressed recombinant enzymes and are currently working to fully characterize the enzymatic activity towards protein and peptide substrates as well as the stability defects of Y835C. In characterizing the variants, we have shown that none of them display issues with dimerization by size-exclusion chromatography, and we have shown the variants have lowered glycosyltransferase activity *in vitro*. Defining which OGT-CDG variants are defective in donor Km may help to define those patients that are more likely to be responsive to substate enhancement therapy.

(P-065) Identifying α2,3 and α2,6-linked Sialic Acid Isomers in N-glycans Present in Human Prostate Cancer Tissues

Kameisha Rashford[1], Caroline Kittrel[1], Bettina F. Drake[2], Peggi M. Angel[1], Joseph E. Ippolito[3], Richard R. Drake[1]

[1]*Department of Pharmacology and Immunology, Medical University of South Carolina, Charleston, SC, USA,* [2]*Institute of Public Health, Washington University School of Medicine, St. Louis, MO, USA,* [3]*Department of Radiology, Washington University School of Medicine, St. Louis, MO, USA,*

Prostate cancer (PCa) remains a major global health challenge, with a notable rise in advanced-stage cases over the past decade, and projections for both incidence and mortality rates are expected to double by 2040. There is an urgent need for new biomarkers to improve early detection, prognosis, and treatment strategies for PCa, thus N-linked sialylated glycans were evaluated in a large cohort (n = 450) of non-metastatic human PCa tissues using matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) workflows. In humans, α2,3 and α2,6 isomers of N-acetylneuraminic acid are the most abundant species detected in sialylated N-glycans. The presence of α2,6 sialylated N-glycans are well-established markers for PCa aggressiveness, while α2,3 sialylation is understudied. Distinguishing between these isomers is important due to their distinct chemical and biological properties, which can provide deeper insights into disease pathology. However, differentiating between the two while preserving histology and spatial distribution is a challenge. Here, we combined a double amidation click-chemical treatment of tissues targeting α2,3 and α2,6 sialic acid isomers with MALDI-MSI to detect their associated glycan structural classes. For each sample, the relative intensities of 60 sialylated N-glycans were summed and percentages determined for each glycan specie. For bi-antennary N-glycans, the percent of singly sialylated N-glycans were 18% for α2,3 and 25% for α2,6 linkages. Doubly sialylated bi-antennary N-glycans were 5% for α2,3, 13% for α2,6 and 4% for mixed. The most abundant α2,6 N-glycans in this class lacked core fucosylation, while the most abundant α2,3 N-glycan was a singly sialylated core fucose specie. For singly sialylated tri-and tetra-antennary N-glycans, the percentage of α2,3 and α2,6 species were equivalent, and core fucosylated species were more abundant. For tri- and tetra-antennary N-glycans with 2–4 sialic acids, there were cumulatively more mixed linkage species than α2,3 and α2,6 only, again with those with core fucosylation being more abundant. The differences in sialylation for each sample are being combined with clinical information to identify potential biomarker candidates. Moreover, as the second amidation reaction incorporates a biorthogonal alkyne into α2,3 linked sialic acids, affinity enrichment of the carrier glycoproteins for subsequent glycopeptidomic analysis is feasible. The combination of

double amidation click-chemical treatment of tissues and MALDI-MSI allows for the differentiation and characterization of sialic acid isomers that were previously difficult to identify, providing a promising advancement in prostate cancer diagnostics.

(P-066) Galectin-3 Targeting Biologic for MASH Therapy

Halima Sultana[1], Khairul Anam[1], Amitava Banerjee[1], Rakin Ahmed[1], Simran Kumar[1], Aditi Banerjee[2], Michael Briggs[3], Hafiz Ahmed[1]

[1]GlycoMantra Inc., bwtech South of the University of Maryland Baltimore County, Baltimore, MD, USA, [2]Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA, [3]Woodland Biosciences, Lafayette, NY, USA,

Metabolic dysfunction-associated steatohepatitis (MASH), previously called non-alcoholic steatohepatitis (NASH) is often linked with disorders that are associated with insulin resistance: obesity, type 2 diabetes, and hypertriglyceridemia. Evidence suggests that galectin-3 (Gal3), a member of the beta-galactoside-binding lectin family, is involved in the development of MASH through up-regulation of inflammatory cell infiltration, pro-inflammatory cytokines, and interaction with cellular receptors: TGF-β receptor, insulin receptor, and pattern recognition receptors. Our scientific premise is that we have developed a potent Gal3 antagonist, GM101, from a natural dietary source. The GM101 selectively targets Gal3 and binds Gal3 with picomolar affinity. We demonstrated Gal3 interaction with TGFβ-RII, and GM101 inhibited this interaction. The efficacy of GM101 to treat MASH was investigated in Streptozotocin-high fat diet (STZ-HFD) mouse model. GM101 significantly decreases steatosis, ballooning degeneration, lobular inflammation, fibrosis, MASLD activity score, and MASH scores compared to the vehicle-treated animals. GM101 also significantly decreases profibrotic factors (Gal3, TGF□, □-SMA) and liver markers (ALT, AST, triglyceride). The efficacy of GM101 to treat fibrosis was also investigated in CCl4 liver fibrosis mouse model and fibrosis was found to be significantly lower in the treatment group. Our results suggest that GM101 can be a significant arsenal against MASH in liver disease.

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Session 2: Glycoconjugates in Infection and Immunity

(Key2-001) Phase Variation in Gardnerella Sialidase & Implications for Bacterial Vaginosis

Sudeshna Saha[1,2,4], Lloyd S. Robinson[3,5], Luis Ramirez-Hernandez[1,2], Christy Armstrong[1,2], Warren G. Lewis[1,2,3], Amanda L. Lewis[1,2,3,†]

[1]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Diego, [2]Glycobiology Research and Training Center, University of California San Diego, [3]Department of Molecular Microbiology and the Center for Women's Infectious Disease Research, Washington University School of Medicine, St. Louis, MO, [4]Present address Sudeshna Saha, Department of Biological Sciences, SRM University-AP, Amaravati, Andhra Pradesh, India, [5]Present address: Omniose LLC, St. Louis, MO,

[†]Presenting Author

Bacterial vaginosis (BV) is a condition characterized by low levels of lactobacilli and overgrowth of diverse anaerobes. The condition is common, occurring in more than a quarter of women. It has been associated with a multitude of adverse reproductive and sexual health outcomes. Sialidase enzyme activity in vaginal fluids is believed to be conferred by bacteria and can be used to diagnose BV. We recently showed that the epithelial glycan landscape in BV is fundamentally altered by sialidase activity, with sialylated N- and O-glycans largely absent compared to samples without BV. Blinded image-scoring analysis of transmission electron micrographs showed that the cellular glycocalyx was visibly diminished in BV. *Gardnerella* is one of the most prevalent and abundant sialidase-producing genera in BV. Treatment of normal (no BV) human epithelial cells with *Gardnerella* sialidase was sufficient to generate BV-like glycan phenotypes. Understanding of *Gardnerella* pathogenesis in BV has been limited by the lack of a bacterial genetic system to enable a 'molecular Koch's postulates' approach using wild type and mutant *Gardnerella*.

Here we take advantage of phase variation in the *nanH3* gene encoding *Gardnerella* sialidase. We show that a polycytosine tract of variable length switches the gene in- and out-of-frame leading to functional changes in sialidase activity. We generated polyC length variants within the same strain background and examined the impact on host-microbe interactions during in vitro and in vivo models. Sialidase is encoded in only a subset of strains, suggesting it is not obligatory for *Gardnerella* to establish colonization in BV. Our data show that *Gardnerella* who do encode an in-frame (phase-ON) *nanH3* sialidase gain new and improved capabilities in carbon metabolism, epithelial interactions, and vaginal colonization compared to variants in the same strain background whose *nanH3* gene is out of frame (phase-OFF). Prior clinical literature shows that among individuals with BV, higher sialidase activity is *associated* with miscarriage, late pregnancy losses, and preterm birth. Here we demonstrate *causal*

roles for *Gardnerella nanH3* sialidase in experimental models of BV that might help explain this clinical picture. Together, the data support the hypothesis that *nanH3* may fuel a virulent subtype of *Gardnerella* that is more likely to trigger adverse events during human reproduction.

(Key2-002) Dress the part: bacterial glycans as dynamic virulence factors and targets for protective antibodies

Nina van Sorge[1]

[1]*Amsterdam University Medical Center,*

Staphylococcus aureus and *Streptococcus pyogenes* are opportunistic pathogens that represent a major public health concern globally. Both pathogens have the ability to cause a wide range of severe life-threatening clinical infections combined with inadequate and even declining prevention and treatment options due to development of antimicrobial resistance. Moreover, vaccine attempts have been frustrated and largely unsuccessful due to a lack of knowledge regarding correlates of protection.

Bacterial glycans are prime targets for some of our most successful childhood vaccines, e.g. meningococci, pneumococci, and *Haemophilus influenzae* b. The surface glycans of *S. aureus* and *S. pyogenes* have yet to be fully characterized and explored for their role in pathogenesis and as possible targets for therapeutic antibodies as well as vaccines. Structural variation and dynamic regulation of the main *S. aureus* and *S. pyogenes* surface glycans, wall teichoic acids (WTA) and Group A Carbohydrate, respectively, impacts molecular host-pathogen interactions and responses.

As examples of new findings related to *S. aureus*, we have identified human Langerin, a C-type lectin receptor unique to skin epidermal Langerhans cells (LCs), as a receptor for β-linked N-acetylglucosamine modifications on *S. aureus* WTA. Recognition by langerin has consequences for induced immunological responses both *in vitro* and *in vivo*[1,2], and is abrogated by the presence of common single-nucleotide polymorphisms in the ligand binding domain of langerin[3]. Second, we have developed and used chemically-synthesized polyribitol-phosphate molecules to dissect interactions between WTA and human antibodies[4]. With these new glycobiology reagents, we have mapped the WTA antibody repertoire in plasma from healthy individuals and patients with culture-confirmed bacteremia to reveal a critical role for WTA-specific IgM in host defense against severe *S. aureus* infections[5]. This finding challenges current IgG-focusing strategies in antibody-based therapies and vaccine development for *S. aureus*. Finally, we employed fully-defined synthetic WTA molecules to unravel the structural details of the interaction with WTA-specific monoclonal antibodies[6] and identify new human glycan-specific monoclonal antibodies through single-cell isolate of specific memory B cells. Our work illustrates how a multidisciplinary approach, including bacterial genetics, microbiology, immunology, and glycobiology, can help unravel the molecular interplay between bacterial glycan variants and host immunity to advance the development of new antimicrobial therapies and vaccines.

References

Hendriks A, van Dalen R, Ali S, et al. Impact of Glycan Linkage to *Staphylococcus aureus* Wall Teichoic Acid on Langerin Recognition and Langerhans Cell Activation. *ACS Infect Dis*. 2021:**7**(3):624–635.

van Dalen R, De La Cruz Diaz JS, Rumpret M, et al. Langerhans Cells Sense *Staphylococcus aureus* Wall Teichoic Acid through Langerin To Induce Inflammatory Responses. *mBio*. 2019:**10**(3).

van Dalen R, Fuchsberger FF, Rademacher C, van Strijp JAG, van Sorge NM. A Common Genetic Variation in Langerin (CD207) Compromises Cellular Uptake of *Staphylococcus aureus*. *Journal of innate immunity*. 2020:**12**(2):191–200.

van Dalen R, Molendijk MM, Ali S, et al. Do not discard *Staphylococcus aureus* WTA as a vaccine antigen. *Nature*. 2019:**572**(7767):E1–E2.

Hendriks A, Kerkman PF, Varkila MRJ, et al. Glycan-specific IgM is critical for human immunity to *Staphylococcus aureus*. *bioRxiv*. **2023**: 2023.2007.2014.548956.

Di Carluccio C, Soriano-Maldonado P, Berni F, et al. Antibody Recognition of Different *Staphylococcus aureus* Wall Teichoic Acid Glycoforms. *ACS Cent Sci*. 2022:**8**(10):1383–1392.

(Key2-003) Group B Streptococcus (GBS) sialic acid capsule blunts mast cell activation through Siglec 9

Irina Miralda[1], Austyn Orvis[2], Michelle Coleman[2], Alyssa Brokaw[2,6], Amanda L. Lewis[3], Nyssa Samanas[1], Adrian M. Piliponsky[1,4,5,6], Lakshmi Rajagopal[2,4,6]

[1]*Center for Immunity and Immunotherapies,* [2]*Center for Global Infectious Disease Research, Seattle Children's Research Institute, Seattle, Washington, USA,* [3]*Department of Ob/Gyn and Reproductive Sciences, University of California, San Diego, California, USA,* [4]*Department of Pediatrics,* [5]*Department of Pathology,* [6]*Department of Global Health, University of Washington, Seattle, Washington, USA,*

Group B *Streptococcus* (GBS) are a leading cause of preterm births, stillbirths and neonatal disease. These bacteria also cause infections in human adults that include normal, elderly and immunocompromised adults. The surface capsular polysaccharide (CPS) is critical for GBS host immune evasion. The GBS capsule displays terminal sialic acids that suppress host innate immune responses by engaging host inhibitory receptors such as the Sialic acid-binding immunoglobin-like lectin- 9 (Siglec-9). Previous studies from our group showed that human mast cells express Siglec-9 and that engaging Siglec-9 results in reduced mast cell degranulation, and chemokine release *in vitro*. As human mast cells express Siglec-9, we hypothesized that sialic acids on the GBS capsule may engage Siglec-9 and impair the ability of mast cells to initiate an immune response against GBS. Consistent with this hypothesis, mice expressing Siglec 7 and 9 exhibit increased GBS burden post infection and this was also observed in mice where only on mast cells express Siglec 9. Further studies to understand how GBS capsule engagement of Siglec 9 hampers mast cell function is ongoing and will provide the foundation to harness mast cell directed therapies to prevent GBS infections.

(Key2-004) Glycoconjugate Processing by Human Gut Microbial Enzymes

Matthew R. Redinbo[1]

[1]*University of North Carolina at Chapel Hill,*

The human gut microbiome plays critical roles in physiological homeostasis, transitions to disease, and therapeutic outcomes. While microbiome sequencing has driven our revolutionary understanding of these effects, specific microbial enzyme families or subfamilies are responsible for producing the molecular consequences that we measure. Here, the identification of individual bacterial proteins from the complexity of human fecal material is presented, along with a pipeline for connecting them to health and disease. The glucuronide conjugates of drugs, hormones and neurotransmitters are activated in the intestinal lumen by a complex family of bacterial beta-glucuronidases that has been connected to health, disease, and variable treatment outcomes. Methods to identify candidate enzyme families from gut microbiome whole genome metagenome sequencing data will be presented, and then combined with ex vivo activity data, targeted metabolomics, and activity-based probe-enabled proteomics to pinpoint specific enzymes that catalyze distinct health-related reactions within complex human fecal material. Examples related to gut-brain axis and variable outcomes with human therapeutics will be presented, and extensions to other microbial enzymes families that process complex glycoconjugates will also be discussed. The goal of this research effort is to pinpoint the microbial activities that promote or detract from human health through their metabolism of simple and complex carbohydrates.

(PT-009) M2 macrophages exhibit enhanced efferocytosis via the inhibitory receptor CD22

Emily N. Kukan[1], Gabrielle L. Fabiano[1], Kevin A. Telfer[1], Julie Y. Zhou[1], Corwin M. Nycholat[2], James C. Paulson[2], Brian A. Cobb[1]

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

Macrophages are cells of the innate immune system capable of adopting a variety of phenotypes and functions in response to environmental cues. In tissue-resident macrophage populations, these phenotypes are known to vary based on the immune tone of the tissue. Likewise, tissue glycosylation also varies, and these glycosylation patterns can influence the immune tone of a tissue. Several reports from our laboratory indicate that removal of α2,6-linked sialylation from tissue environments causes a pro-inflammatory shift in tissue-resident macrophage phenotype. Therefore, we hypothesized that macrophages express sialic acid-binding ig-like lectin 2 (CD22), an inhibitory receptor restricted to α2,6-linked sialic acid recognition previously thought to be expressed only in B cells, where it prevents self-reactive B cell receptor (BCR) responses through recognition of sialylated antigens. Here, we report that CD22 expression in macrophages is strongly associated with an M2, anti-inflammatory phenotype and primarily driven by IL-4. Similarly, CD22 expression is higher in tissue-resident macrophage populations that skew toward an M2-like phenotype, such as liver and lung macrophages. These tissues also show a high degree of α2,6-sialylation, indicating an abundance of CD22 ligand in these environments. In B cells, CD22 is known to be a potently endocytic receptor. Corroborating this, we have found that $CD22^+$ macrophages show enhanced endocytosis of sialylated glycoproteins. Finally, we report that CD22 enhances efferocytosis, a process through which macrophages endocytose apoptotic cells and prevent the exacerbation of inflammation. Thus, we conclude that CD22 expression is a feature of a subset of M2 macrophages involved in the maintenance of tissue homeostasis through the clearance of α2,6-sialylated "self" debris, particularly apoptotic cells such as neutrophils. Ongoing work in our laboratory aims to understand the downstream signaling initiated by CD22 in macrophages and the upstream control of its expression in these populations.

(PT-011) Inflammation-induced antibody sialic acid acetylation disables protection against intracellular infection

April Smith[1,2,†], Kubra Gokce Tezel[1,2,†], Aminul Islam[1,2,†], Stephanie Seveau[3], Sing Sing Way[2], John Erickson[1,2,‡]

[1]*Division of Neonatology and Pulmonary Biology, 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]*Department of Microbial Infection and Immunity, Ohio State University, Columbus, OH, USA,*

[†]Authors contributed equally
[‡]Presenting author

Antibodies ineffectively protect against some infections caused by intracellular pathogens. We recently demonstrated that pregnancy modifies sialic acid (Sia) on antibody N-glycans to enable maternal and neonatal host defense against the prototypical intracellular bacterium *Listeria monocytogenes* (Lm). During pregnancy, maternal Sia acetyl esterase (SIAE) deacetylated Sia on IgG variable region N-glycans. However, it is unknown how Lm infection outside of pregnancy leads to the production of non-protective IgG possessing acetylated Sia. Utilizing Lm mutants with differential access to cellular compartments, we now show that in virgin mice bacterial cytoplasmic entry triggers production of the Th1 cytokine IL-12 to elicit IgG possessing 9-*O*-acetyl-Sia. In contrast, phagosome-confined Lm unable to escape into the cytoplasm produces minimal IL-12, generates IgG with non-acetylated Sia, and thus bypasses the necessity for maternal SIAE-mediated Sia deacetylation for neonatal protection against Lm infection. Neonates require the Sia receptor CD22 to recognize deacetylated Sia on maternal IgG. Addtionally, IL-12 promotes IgG 9-*O*-acetyl-Sia following recombinant antigen immunization targeting critical Lm virulence factors. Thus, IgG Sia acetylation also impairs antibody-mediated immunity in the setting of protein-based immunization strategies. These findings highlight inflammation-induced IgG Sia acetylation as a critical element controlling host defense and offer new avenues for the prevention of intracellular infections by rationally targeting the N-glycans on pathogen-specific antibodies.

(PT-013) Sialidase enzymes derived from bacterial vaginosis associated bacteria may impair sperm function by remodeling the sperm glycocalyx

Sarah Dohadwala[1], Purna M. Shah[1], Maura K. Farrell[1], Joseph A. Politch[2], Kevin Whaley[3], Deborah J. Anderson[1,2], Catherine E. Costello[1]

[1]*Boston University, Boston MA,* [2]*Boston University Medical School, Boston MA,* [3]*ZabBio Inc., San Diego CA,*

Bacterial vaginosis (BV), a dysbiosis of the vaginal microbiome, affects approximately 23 to 29 percent of women worldwide and is associated with several adverse health outcomes including preterm birth, subfertility, and sexually transmitted infections (STI). BV-associated bacteria, such as *Gardnerella vaginalis*, are known to cause epithelial damage and degradation of the vaginal mucosa through the activity of sialidase enzymes that remodel the epithelial glycocalyx and metabolize mucin glycoproteins. This damage to the vaginal glycocalyx creates an inflammatory environment which likely contributes to adverse health outcomes. However, whether BV-associated glycolytic enzymes can also damage sperm during their transit in the reproductive tract has not yet been determined. Here, we show that sialidase-mediated glycocalyx remodeling of human sperm increases sperm susceptibility to innate immune damage within the female reproductive tract. In particular, we report that upon exposure to physiologically relevant amounts of sialidase enzymes, desialylated human sperm demonstrate increased susceptibility to complement lysis (~2.5-fold) and agglutination (~2-fold). Our results demonstrate a potential mechanism by which BV glycolytic enzymes may affect sperm survival and function and thereby contribute to adverse reproductive outcomes such as subfertility.

(PT-015) Mannosylated Protein Turnover Links Mrc1 to Inflammation and Sepsis

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

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

As nascent blood glycoproteins circulate, their glycan linkages can be progressively hydrolyzed by exo-glycosidases. This form of molecular aging exposes underlying cryptic glycan ligands of various endocytic lectin receptors. In cases analyzed thus far, the rate of glycan linkage hydrolysis is inversely proportional to glycoprotein half-life and abundance in circulation. The rate of endocytic clearance, versus the rate of synthesis, most rapidly controls the abundance and function of circulating glycoproteins.

Pathogens target this host mechanism in multiple diseases including sepsis and colitis with significant impacts on pathogenesis and host outcome. We have herein applied an optimized methodology previously developed to identify physiological ligands, and thereby the functions, of individual mammalian lectin receptors operating in the vasculature. In this study we report a significant and previously unknown repertoire encompassing mannosylated glycoprotein ligands with abundance in circulation responsive to endocytic Mrc1 lectin receptor function. Concordant findings in Mrc1 deficiency included the accumulation of mannosylated Renin and Angiotensin Converting Enzyme linked to elevations of systolic and diastolic blood pressure and cardiovascular fibrosis, and the accumulation of mannosylated Myeloperoxidase with elevated lipid oxidation, vascular inflammation, and leukocyte infiltration. Mrc1 is expressed in multiple cell types including the brain where multiple anatomic regions underwent a significant increase in neurological markers of inflammation associated with abnormal behavior and locomotion. Multiple phenotypes of mice lacking Mrc1, missed in previous studies present as a progressive age-associated low grade but significant increase in inflammation. Mrc1 deficiency further reduced viability in the context of sepsis with findings of Mrc1 proteolysis and a significant increase in circulating mannosylated proteins. Remarkably, we found that human sepsis, previously linked by others to elevations of Mrc1 proteolysis and abundance in circulation, was also linked to elevations in circulating mannosylated protein levels. In comparing mouse Mrc1 deficiency with human sepsis, we found significant concordance in the activation of functional pathways establishing that the inflammatory signaling pathways common to sepsis is represented by a defect of mannosylated protein turnover by Mrc1.

(P-067) Altered composition of glycan microdomains of HIV-1 envelope glycoprotein (Env) is sufficient for viruses to escape recognition by broadly neutralizing antibodies (BnAbs)

Qing Wei[1], Zhengrong Yang[2], Barbora Knoppova[1], Shihong Qiu[1], Qiao Shang[1], Audra Hargett[2], Stacy Hall[1], Zina Moldoveanu[1], Janet Yother[1], Todd J. Green[1], Matthew B. Renfrow[2,†], Jan Novak[1,†]

[1]*Departments of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA,* [2]*Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA,*

[†]Co-senior authors

HIV-1 Env, a trimer of gp120 and gp41 dimers, is the sole target for BnAbs. *N*-glycans represent > 50% of gp120 molecular mass, forming a "glycan shield" that is involved in virus-cell interactions, immune recognition, and immune evasion. The Env glycan shield is highly variable due to the diversified Env sequences and cell-specific glycosylation mechanisms. We postulate that Env glycan shield can be defined by the distinct structural glycan microdomains that complement the function and stability of the protein domains of the Env trimer. To better understand the functions of Env glycan microdomains, we have used various means to alter glycosylation of HIV-1 Env.

We used Env from an immune-escape clade B HIV-1 (WEAU-d391; *WT*) and its variants modified by removing i) a single *N*-glycosylation site (NGS) in the V2 region (*N188-Mut*), ii) two NGS (N273 and N463) that restrict accessibility to the CD4 binding site (*CD4bs-Mut*), or iii) all three NGS (*3NGS-Mut*). Env-pseudotyped viruses and the corresponding recombinant gp120 trimers (rgp120) were produced in FreeStyle 293-F (FS) or Expi293F (XP) cells. TZM-bl reporter cells were used to determine the infectivity of the Env-pseudotyped viruses and their sensitivity to BnAbs, including those specific for the CD4bs (VRC01) or the apex of the Env trimer (PG16, PGT145). PGT122 that targets the C3 region around N332 served as a control BnAb as its neutralizing activity is generally not impacted by Env glycan variations. We analyzed rgp120 glycosylation by profiling total and site-specific *N*-glycan content. Structural modeling was used to interpret the glycomics and functional data.

The tested NGS mutations did not abolish the ability of viruses to infect the reporter cells. However, infectivity of the *WT* virus was reduced when produced in XP cells compared to FS cells. XP-produced Env had an elevated content of high-mannose glycans, indicating a cell-specific differential glycosylation of the same Env amino-acid backbone. *CD4bs-Mut* and *3NGS-Mut* viruses were ~15-fold more sensitive to VRC01, as expected, irrespective of the producing cells. *WT* viruses produced in FS cells were highly sensitive to PGT145, whereas *N188-Mut* viruses from the same cells were > 80-fold less sensitive, indicating that N188 glycans impact the apex glycan microdomain. PG16 effectively neutralized *WT* viruses produced in FS cells, whereas *N188-Mut* viruses were resistant to PG16. Moreover, all viruses produced in XP cells were less sensitive to PGT145 and resistant to PG16. PGT122 neutralized all viruses similarly. Site-specific glycosylation analyses revealed elevated content of high-mannose vs. complex glycans at N160, the key glycan impacting binding of apex-targeting BnAbs.

Our results revealed that alteration of Env glycan microdomains impacts HIV-1 infectivity and sensitivity to BnAbs. These findings may have implications for HIV-1 immune escape *in vivo*.

(P-068) Glycoproteomics of envelope glycoproteins reveals shared principles across diverse viruses

Joel D. Allen[1], Maddy Newby[1], Yasunori Watanabe[1], Max Crispin[1]

[1]*School of Biological Sciences, Highfield Campus, University of Southampton, UK, SO17 1BJ,*

Almost all pandemic viruses utilize densely glycosylated envelope glycoproteins to permit cellular infection. The crucial role that these glycoproteins perform is highlighted by the pervasiveness of neutralizing antibody epitopes across their surface, which also makes them ideal candidates for inclusion in a vaccine. As the N-linked glycans attached to viral glycoproteins are derived from the host, they are subject to the same influences that dictate host N-glycosylation. However, there are multiple additional considerations that are specific to viral N-linked glycan processing. Viral glycoproteins contain N-glycan attachment sequons at a significantly higher density than host glycoproteins, and these host-derived N-glycans are able to thwart antibody recognition of more antigenic protein epitopes. We utilize mass spectrometry to determine the site-specific glycosylation of viral glycoproteins to unpick the rules that govern the attachment of viral glycans, this allows for the presentation of native-like epitopes, which is considered desirable in HIV-1 vaccine research, or to engineer non-native glycosylation, which can offer routes to enhanced immunogenicity. This talk will detail the lessons learned from studying the glycan shields of a range of viruses including HIV-1, SARS-CoV-2, influenza, and hepatitis C, including the relationship between glycan shield density and glycan processing, the influences of vaccine candidate glycan underoccupancy on antigenicity and how the glycan shield of viruses can remodel itself in response to interactions with the host immune system. Determining the rules that govern the attachment and processing of N-linked glycans to viral glycoproteins is an important step in the development of viral vaccine candidates.

(P-069) Profiling and Modulating Desialylation Pathway in THP1-Macrophages upon Lipopolysaccharide (LPS) Stimulation

Majdi Aljohani[1], Xue-Long Sun[*,1]

[1]*Department of Chemistry, Chemical and Biomedical Engineering and Center for Gene Regulation of Health and Disease (GRHD), Cleveland State University, Cleveland, OH,*

The cell surface is significantly coated with glycans that are terminated by sialic acids (Sias), a process known as sialylation. Desialylation, on the other hand, is essential for a variety of biological processes and refers to the removal of Sias. Lipopolysaccharide (LPS) induces endogenous sialidase expression in macrophages, which could result in desialylation of the TLR4, thereby initiating the LPS/TLR4 signaling pathway. In this study, we investigated the desialylation process in THP-1 macrophages upon LPS stimulation. Confocal microscopy confirms enhanced Neu1 translocation from lysosomes to the cell surface following LPS stimulation, indicating its involvement in TLR4 desialylation. Surprisingly, sialidase levels significantly increased in the cell culture media, indicating sialidases secretion from THP-1 macrophages as well. Furthermore, lectin blot analysis and flow cytometry showed desialylation of total proteins in LPS-stimulated THP-1 macrophages. The sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) inhibits inflammatory responses by inhibiting sialidase activity. DANA specifically reduces the production of cytokines and reactive oxygen species (ROS) by regulating IKBα phosphorylation, signaling, and subsequent inflammatory pathways. This is the first systematic profiling of desialylation of macrophages upon LPS stimulation, indicating that endogenous sialidase expression and secretion are involved in the LPS/TLR4 signaling pathway and subsequent biological processes and cellular functions as well.

(P-070) Understanding the role of fucose in Pertussis Toxin binding

Rohit Sai Reddy Konada[1], Aurora Silva[1], Jennifer J. Kohler[*,1]

[1]*Department of Biochemistry, UT Southwestern Medical Center, Dallas, TX 75390 USA,*

Pertussis (whooping cough) is caused by the bacterium *Bordetella pertussis*. This bacterium produces a number of virulence factors and pertussis toxin (PT) is responsible for many of the disease symptoms. PT is a AB5 type toxin, consisting of a single catalytic A subunit with ADP-ribosyltransferase activity and five heteromeric B subunits. The pentameric B subunits contain binding sites that bind to the cell surface glycans on various human cell types. The B subunit acts as a delivery system leading to internalization of the holotoxin and subsequent host cell intoxication. In the current study we identified that the absence of fucose on cell surfaces was associated with increased binding of PT. By using pharmacological inhibitors, we found that sialylated

N-glycans are important in PT binding and the inhibition of fucosylation enhanced the binding of toxin to HBEC-3KT cells. Mutant cell lines and genetic knock out experiments showed similar effects of fucose on toxin binding to CHO cells and Colo 205 cells. Further, we identified that knocking out FUT3 or FUT8 in Colo 205 cells greatly increased PT binding, suggesting the linkage of fucose involved in this effect. Our study highlights the role of cell surface fucosylation in PT binding to mammalian cells.

(P-071) Sugar import diversifies Klebsiella pneumoniae capsular polysaccharide chain length and reduces hypermucoviscosity

Saroj Khadka[1], Laura A. Mike[†,1]

[1]*Department of Medicine/Division of Infectious Diseases, University of Pittsburgh, Pittsburgh, PA, USA,*

[†]Presenting Author

Capsular polysaccharides (CPS) are crucial for the virulence and fitness of *Klebsiella pneumoniae,* a Gram-negative bacterium responsible for a wide range of infections. Modifications to various CPS properties, such as chain length, can further enhance the virulence potential of *K. pneumoniae*. Recent studies have shown that the hypermucoviscosity (HMV) phenotype, characterized by increased tackiness of the bacterial colonies, is caused by longer, uniform-length CPS chains. Importantly, HMV confers hypervirulence to *K. pneumoniae* and promotes invasive infections. Our previous work demonstrated that culturing *K. pneumoniae* in pooled human urine versus a nutritionally rich medium drastically modifies CPS chain length distribution. This study aimed to identify specific nutrient signals and regulatory mechanisms underlying environment-dependent CPS chain length modulation. Using defined minimal growth medium supplemented with sugars at varying concentrations, we tested the hypothesis that extracellular nutrients, specifically sugars, induce changes in *K. pneumoniae* CPS chain length diversity distinct from CPS abundance. We employed sedimentation resistance assay, uronic acid quantification and glycostaining to assess HMV, CPS abundance and chain length distribution in the various sugar-supplemented medium, respectively. Our results demonstrated that ten metabolizable and non-metabolizable sugars included in the study significantly suppressed *K. pneumoniae* HMV, which correlated with unimodal distribution of CPS chains. These data indicated that sugar transport, not catabolism, is a cue regulating CPS chain length and HMV. Moreover, sugar supplementation significantly downregulates *rmpD*, which regulates CPS chain length and, subsequently, HMV. To further elucidate the mechanism linking sugar import to *rmpD* transcription and HMV, we screened a KPPR1 transposon library and employed RNAseq to identify genes required for sugar-mediated HMV suppression. Finally, non-HMV *K. pneumoniae* pre-cultured in sugar-supplemented medium showed reduced association and invasion of macrophages compared to HMV *K. pneumoniae* cultured in amino acid-supplemented medium. Collectively, these findings reaffirm the impact of CPS chain length on *K. pneumoniae* HMV and suggest that the import of host-derived sugars could regulate *K. pneumoniae* HMV during an infection to optimize niche-specific fitness. Further elucidation of the niche-dependent chain length and HMV regulation mechanism *in vivo* will reveal how bacterial adaptation of surface-exposed polysaccharides alters pathogenesis and infection outcomes.

(P-072) Macrophage Siglecs- a promising immune checkpoint target for pancreatic cancer patients with high levels of ST6GAL1 sialyltransferase

Barnita Haldar[1], Susan L. Bellis[1]

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

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a poor prognosis. A key factor contributing to the lethality of PDAC is immune evasion. Tumor cells suppress anti-tumor immunity by activating immune checkpoint proteins on various immune cells including macrophages. Siglec receptors are one of the main checkpoint molecules on macrophages. The ligand for Siglecs is sialic acid. To effectuate immune suppression via Siglecs, PDAC cells increase their surface sialylation by upregulating the expression of sialyltransferases such as ST6GAL1. ST6GAL1 adds an α2,6-linked sialic acid to N-glycans. Both α2,6 sialylation and ST6GAL1 expression are markedly increased in PDAC cells. Our prior studies revealed potent tumor-autonomous functions for ST6GAL1. Our new unpublished results suggest that the tumorigenic effects of ST6GAL1 are also driven by creating sialoglycan ligands for macrophage Siglecs. Macrophages that are polarized to an immunosuppressive M2 phenotype are key contributors to PDAC progression. The goal of our study is to determine whether macrophage Siglecs are promising therapeutic targets for preventing M2 polarization, thus restoring anti-tumor immunity and preventing PDAC progression. We investigated macrophage polarization in our genetically engineered mouse models of PDAC, utilizing either pancreas-specific knock-in of oncogenic K-ras (KC mouse) or K-ras in combination with ST6GAL1 knock-in (KSC mouse).

Flow cytometry and immunohistochemistry showed an increased number of M2 macrophages in KSC vs. KC pancreata. Preliminary data from single-cell RNA sequencing showed an increase in M2 gene expression in macrophages from KSC mice. Next, macrophages were co-cultured with PDAC cells with differential ST6GAL1 expression for 72 hours to induce macrophage polarization. Macrophages co-cultured with ST6GAL1-overexpressed PDAC cells showed increased expression of M2 markers, suggesting that ST6GAL1-overexpressed PDAC cells induce macrophage polarization to the M2 phenotype. However, adding Siglec-blocking antibodies reversed the ST6GAL1-mediated M2 polarization in co-culture assays, indicating that the immune suppressive effects of ST6GAL1 are mediated through Siglec signaling. Finally, we evaluated the phagocytotic capacity of macrophages in co-culture assays. Phagocytosis is an established functional read-out for an anti-tumor behavior of macrophages. Phagocytosis was measured using a standard assay based on the uptake of fluorescent beads. The phagocytotic capability of macrophages was suppressed by ST6GAL1-overexpressed PDAC cells and recovered by Siglec blocking antibody. Overall, our data demonstrate that α2,6 sialic acids on PDAC cells engage with Siglecs on macrophages to induce polarization of macrophages into an immunosuppressive M2 phenotype. Collectively, these results reveal a potential mechanism for targeting Siglecs as a promising immune checkpoint therapy for PDAC patients with high levels of ST6GAL1.

(P-073) Th17 cells facilitate the HIV Env glycopeptide-induced adaptive humoral immune responses

Zahra Nawaz[1], Trevor Adams[1], Mukaddes Sena Cinar[1], Fikri Y. Avci[1]

[1]*Department of Biochemistry, Emory Vaccine Center, Emory University School of Medicine Atlanta, GA 30322,*

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus-1 (HIV-1) remains among the most common causes of death from an infectious organism. However, a protective HIV vaccine has not as yet been developed. Thus, there is an urgent need for the generation of a protective HIV vaccine. Recent studies have demonstrated critical roles for CD4+ helper T cells in driving antibody subclass switching, affinity maturation, and effector function of antibodies to HIV. Particularly, the generation of bNAbs requires affinity maturation and somatic hypermutations requiring CD4+ T cell help. Therefore, recruiting T cells to induce high-affinity, long-lasting, and protective antibody response against the gp120 glycan shield is critical. Towards illuminating the mechanisms of T cell-mediated humoral immune responses induced by the HIV-1 envelope glycoprotein gp120, we previously reported that a CD4+ T cell repertoire recognizes a glycopeptide epitope (GpepIP) on gp120 presented by the MHCII pathway. This glycopeptide is strongly immunogenic in eliciting glycan-dependent cellular and humoral immune responses. We also observed that glycopeptide immunization of mice drives a Th17 differentiation with potent Env trimer humoral immune responses. Glycopeptide immunization elicits booster IgG response targeting gp120 glycan-epitopes shared by HIV Env immunogens across clades. Our findings indicate a role for Th17 cells for the gp120 glycopeptide-specific adaptive humoral immune responses. Elucidating the mechanisms of Th17 helper T cell stimulation by HIV-1 Env glycopeptide epitopes may lead to major advances in the future development of glycan-based vaccines against HIV.

(P-074) Modulating glycosylation sequons on Mycobacterium tuberculosis Ag85 protein complex to exploit their immunological potential as an mRNA-based TB vaccine

Mukaddes Sena Cinar[1], Aidan Keelaghan[1], Trevor Adams[1], Zahra Nawaz[1], Ahmet Ozdilek[1], Fikri Avci[1]

[1]*Department of Biochemistry, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322,*

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). The only available licensed vaccine is the Bacillus Calmette-Guérin (BCG) vaccine, a live attenuated whole-cell vaccine that poorly protects against pulmonary TB in adults and adolescents. Nucleic acid vaccines are an innovative approach to vaccination that aims to generate an adaptive immune response against the encoded genetic material. MVA85A and AERAS-402 are two mRNA vaccines targeting the Ag85 complex of Mtb composed of three homologous, immunodominant surface immunogens (Ag85A, Ag85B, and Ag85C). However, in Phase II clinical trials, both vaccines failed to generate protection against Mtb. We speculated that the Ag85 complex expressed in the host went through glycosylation following nucleic acid vaccine administration, impairing their immunogenicity and antigenicity. We demonstrated that Ag85A and Ag85B expressed in mammalian cells are N-glycosylated, consequently dampening their immunogenicity and antigenicity compared to the Mtb-expressed non-glycosylated native proteins. Removing these N-glycosylation sites through site mutations rescued the dampened immune response. Our findings suggest that N-glycosylation of target antigens expressed by mRNA vaccines in hosts may lead to variable immunogenic and antigenic responses. Implications of this research would help dictate novel nucleic acid vaccine technology and designs to modulate potential post-translational modifications of non-viral pathogenic antigens and their accompanying immune responses.

(P-075) Alcohol induces altered glycans that compromise immune function, increase adhesion of bacteria and protect benign cancer from immune surveillance

Pi-Wan Cheng[1], Derrick Samuelson[1]

[1]*University of Nebraska Medical Center, Omaha, NE,*

Alcohol is known to increase infections and cancer fatality. But the mechanism is ill-defined. Recently, we found that alcohol prevented Golgi targeting of glycosylation enzymes to giantin site in human airways epithelial cells, which led to loss of core 2 enzymes and shifted Golgi targeting of other enzymes to Golgi Matrix Protein130-Golgi Reassembly Stacking Protein 65 site, resulting in the formation of α2-6sialo mucin O-glycans that killed U937 macrophages. Here, we extended this observation to show that alcohol enhanced adhesion of *Streptococcus pneumoniae* and *Klebsiella pneumoniae* to human airways epithelial cells, which were inhibited by SNA-I lectin and αmethyl mannoside, respectively. Alcohol also induced in benign colon cancer FET cells α2-6sialo mucin O-glycans that killed U937 macrophages, which was prevented by SNA-I lectin. Further, U937 macrophages were killed after exposure to malignant colon cancer HCT116 cells, which was also prevented by pretreatment with SNA-I lectin. The results suggest that alcohol increases infections by compromising immune function and increasing adhesion of pathogens and promotes cancer progression by protecting benign cancer from immune surveillance.

(P-076) Constructing Glucose-6 and Mannose-6 oxidases for next-generation conjugate vaccine production

Aidan Keelaghan[1], Trevor Adams[1], Afaq Niyas[1], Fikri Avci[1]

[1]*Emory University: School of Medicine,*

Pneumococcal pneumonia hospitalizes approximately 150,000 people in the US each year, with a 5–7% mortality rate, and is estimated to kill more than 1,000,000 children annually. *Streptococcus pneumoniae* (Spn) serotypes are encased in a dense matrix of distinct capsular polysaccharides (CPS), pivotal in their pathogenicity. CPSs must be covalently conjugated to protein carriers to generate T cell-dependent glycoconjugate vaccines eliciting an adaptive immune response. The functionalization of CPS required for conjugating to carrier proteins predominantly relies on their chemical oxidation. However, chemical oxidation is empirical, loosely controlled, and structurally damaging, leading to significant structural alterations and variability in CPS antigenicity. An alternative to chemical oxidation is using an enzyme, galactose oxidase (GOase), that can oxidize the primary alcohol on terminal non-reducing ends of galactose residues in CPS. We demonstrated that this highly selective oxidation significantly improves the chemical and immunological characteristics of glycoconjugate vaccines. However, GOase oxidizes Spn serotypes only with galactose non-reducing ends, limiting its applicability across strains and serotypes. Constructing GOase-like enzymes specific for glucose and mannose terminal sugars would significantly expand the CPS repertoire that can be chemoenzymatically oxidized for use in glycoconjugate vaccines. Thus, the development of oxidases specific for terminal glucose or mannose residues on CPSs can be revolutionary in conjugate vaccine design. Utilizing state-of-the-art *in silico* and *in vitro* enzyme engineering platforms, we investigate the rational design of galactose oxidase-like enzymes specific for glucose or mannose. The newly engineered mannose and glucose oxidases will expand the range of clinically important Spn serotypes to be included in rationally designed glycoconjugate vaccines. Establishing their enhanced efficacy and superior biophysical and immunological properties compared to traditionally prepared formulations will lay the foundation for developing next-generation glycoconjugate vaccines.

(P-077) Oxidative Release of Natural Glycans (ORNG): Unraveling the Mechanism for Rapid N-Glycan Glycomics Analysis

Qing Zhang[1], Yi Lasanajak[2], Xuezheng Song[1,2]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, United States,* [2]*Emory Glycomics and Molecular Interactions Core, Emory University School of Medicine, Atlanta, Georgia 30322, United States,*

N-glycosylation is an essential post-translational modification with profound implications in various biosynthetic pathways and disease pathology. Previously, we reported the oxidative release of natural glycans (ORNG) using household bleach to produce N-, O-, and glycosphingolipid glycans. In this manuscript, we conducted a detailed mechanistic study on the release of N-glycans and characterized several side products. The mechanistic study sheds light on the development of a method for rapid and specific N-glycomics analysis that can be applied universally to various samples. Application of this method on various beans and human serum demonstrates the efficiency and sensitivity of this inexpensive and unique approach for N-glycomics.

(P-078) Human Siglec-3/Siglec-8 sialoglycan ligands produced in genetically engineered HEK293 cells expressing carbohydrate sulfotransferase CHST1

Anabel Gonzalez-Gil[1], Ronald L. Schnaar[1]

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

Siglecs are a family of sialic acid binding immunoglobulin like lectins, most of which are expressed on immune cells where they modulate the immune responses. Each immune cell carries its own suite of Siglecs which can either drive immune inhibition or immune excitation depending on the Siglec and the ligand it engages. Siglec-8 is expressed on human eosinophils and mast cells in the periphery and microglia in the human brain. It is among the most specific glycan binding proteins, requiring a sialylated sulfated terminal galactose, Neu5Acα2-3(6-SO4)Gal, as a minimum binding determinant. Siglec-3 is more broadly expressed and binds avidly to the same terminal sequence along with some other sialoglycans. Crosslinking Siglec-8 on eosinophils results in apoptosis and on mast cells it inhibits the release of inflammatory mediators, making it an inviting target for anti-inflammatory therapy for atopic diseases. Crosslinking Siglec-3 on microglia reduces phagocytosis. In human tissues, sialylated keratan sulfate chains have been identified as ligands for Siglec-8 carried on different proteins depending on the tissue type it is expressed. In human airway lumen, the glycoprotein DMBT1 is modified with Siglec-8 ligands, whereas in the brain Siglec-8 as well as Siglec-3 ligands are carried on the proteoglycan RPTPζ. Although cross-reactive Siglec-8 and Siglec-3 ligands have been successfully identified and purified from postmortem human tissues and human secretions, stable in vitro expression from human cells had not been established. Primary human cells lost their ability to produce ligand within 2 weeks in culture and stable human cells lines did not make these ligands. To resolve this issue, we used a genetically engineered HEK293 cell line expressing the carbohydrate sulfotransferase CHST1 (Büll et al, *PNAS* 118:e2026102118, 2021). When transiently transfected with DMBT1 or RPTPζ this engineered cell line produces cross-reactive Siglec-8/Siglec-3 ligands DMBT1$^{S3/S8L}$ and RPTP$\zeta^{S3/S8L}$. Expressed ligand is sensitive to sialidase and keratanase I treatment and has an affinity for Siglec-8/3 in the picomolar range similar to endogenous human ligands. At scale expression of the ligands is now feasible for exploring their mode of action in immune regulation.

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(P-079) Endothelial cells mediate IgG glycan remodeling as a function of pregnancy

Leandre M. Glendenning[1], Gracie C. Carlson[1], Megan D. Long[1], Wendy A. Goodman[1], Brian A. Cobb[1]

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

Sialylation of the conserved glycan on the Fc domain of IgG serve as key anti-inflammatory modifications during the host immune response. 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. Additionally, it has been previously reported that IgG sialylation is increased during pregnancy, correlating with remission of rheumatoid arthritis in female patients. 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 recently revealed the existence of a novel FcRn-mediated glycan remodeling pathway in endothelial cells which drives B cell-extrinsic IgG sialylation. Thus, we hypothesized that anti-inflammatory modifications, such as sialylation, are increasingly added to the IgG glycan by endothelial cells during pregnancy. Here, we show evidence that modifications to the IgG glycan are seen in murine pregnancy, strongly correlating with human epidemiologic data. Further, these modifications appear to be dependent on endothelial cells throughout the mother's body. Our findings reveal a close link between endothelial cells and pregnancy that drives changes in IgG glycosylation and function.

(P-080) Inflammatory signaling in endothelial cells drives changes in IgG sialylation

Gracie C. Carlson[1], Leandre M. Glendenning[1], Megan D. Long[1], Austin D. Silva[2], Susan L. Bellis[2], Brian A. Cobb[1]

[1]*Department of Pathology, Case Western Reserve University School of Medicine,* [2]*Department of Cell Developmental and Integrative Biology, University of Alabama at Birmingham,*

A core component of the humoral immune response is IgG, an antibody class distinguishable by its unique heavy chain composition and by its integral role in protective immunity as well as immunologic homeostasis. Glycan composition within the

Fc domain at the conserved and N-glycosylated N297 site is a critical point of regulation that dictates the function of IgG through alterations in Fc domain conformation. Terminal α2,6 sialylation of this glycan by the sialyltransferase ST6Gal1 has been shown to contribute to a homeostatic immune state through suppression of receptor signaling. Epidemiologic data over the past four decades has demonstrated that inflammation is linked to a loss of both sialic acid and galactose residues on IgG, presumably to enhance the pro-inflammatory environment. In our previous and recent work, we have discovered that the endothelium rather than B cells is the dominant location at which IgG is sialylated. However, understanding the regulation of ST6Gal1 and thereby IgG sialylation remains a significant knowledge gap that poses a hurdle in obtaining a broadened understanding of IgG functional regulation. Here, we report that B cells and endothelial cells primarily use different ST6Gal1 promoters. We have also found that inflammatory signaling drives suppression of IgG sialylation via reduced ST6Gal1 expression within endothelial cells but not B cells. These data support the existence of a differentially regulated IgG glycan remodeling pathway in endothelial cells which drives alterations in IgG sialylation within the plasma.

(P-081) Differential Impact of IgG1 Glycoforms on Platelet Activation and Degranulation

Sneha Ratnapriya[1], Robert M. Anthony[1]

[1]*Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School,*

Platelets are established to play a crucial role in hemostasis and thrombosis. Additionally, these cells express immune receptors including FcγRIIa (CD32a), FcεR1α, and FcαR1 (CD89) that bind antibodies IgG, IgE, and IgA, respectively. Recent studies have demonstrated that composition of the single, N-linked glycan on IgG1 exerts profound influence on other FcγR expressing cells, including macrophages and B cells. However, it is not known whether differential glycosylation on IgG influences antibody-mediated platelet activation. To this end, we stimulated platelets with sialylated and asialylated IgG1 immune complexes, measuring alpha and dense degranulation by P-selectin (CD62P) and LAMP3 surface expression, respectively. Surprising, the glycosylation variant complex induced differential platelet activation. Further, we quantified sugar nucleotide levels in platelet releasates after stimulation with sialylated or asialylated IgG1 immune complexes, and found significantly higher sugar release by sialylated IgG1 complexes. Next, we sought to understand the specific receptors required by the different IgG1 glycoforms. We found that blocking FcγRIIa significantly reduced activation by the asialylated IgG1 immune complex, whereas it had a non-significant effect on the activation mediated by the sialylated immune complex. Taken together, this study underscores a role of FcγRIIa receptor engagement in the action of asialylated IgG1 immune complexes, while implicating addition factors in platelet activation by sialylated IgG1 immune complexes. Our results suggest that different IgG1 glycoforms utilize distinct receptors to activate platelets, highlighting the complexity of platelet interactions and the specificity of receptor-glycoform engagements. Understanding the distinct responses of platelets to IgG1 glycoforms illuminates distinct effector pathways of platelets as IgG effector cells. In summary, our research illuminates the intricate dynamics of platelet activation and degranulation in response to different IgG1 glycoforms, further enhances our understanding of platelet biology, and suggests implications for therapeutic strategies in immune-related conditions.

(P-082) Enhanced Synthesis of Sulfated and Polymer-Linkable Mucin Core Glycans

Joseph R. Romeo[1], Katharina Ribbeck[1]

[1]*Massachusetts Institute of Technology, Department of Biological Engineering,*

Mucin glycopolymers, essential components of the protective mucus layer lining epithelial surfaces, present a diverse array of glycan signals that modulate interactions with the microbiome and influence virulence gene expression. These glycans, ranging from simple core structures to complex oligosaccharides, are densely displayed on a protein backbone and often decorated with sulfate, sialic acid, and fucose residues, enhancing their protective and signaling functions. Recent interest in mucin-mimetic materials for studying the molecular basis of mucin-microbe interactions necessitates access to well-defined, polymer-displayable core glycans. While established synthetic routes exist for natural core glycans, scalable methods for preparing derivatives such as sulfated glycans and reducing-end O-glycosides suitable for polymer conjugation remain underdeveloped. To address this need, we have developed an orthogonal protecting group strategy enabling the efficient attachment of selectively sulfated core glycan building blocks to a N-trichloroacetyl galactosamine bearing a flexible silyl aglycone. This approach also features the early incorporation of aryl-protected sulfates via a Sulfur (VI) Fluoride Exchange (SuFEx) reaction, facilitating compatibility with both glycosylation conditions and hydrogenative deprotection. This key advancement allows for the divergent synthesis of both reducing-end and polymer-linkable sulfated glycans. We will present our progress towards the scalable production of sulfated core 1 glycans equipped with O-glycoside handles amenable to polymer incorporation.

(P-083) Glycoengineering Anti-HIV Antibodies To Increase Delivery To The CNS

Samantha Welninski[1], Yuekun Lang[2], Dina Mahmoud[2], Gabrielle Kooi[1], Basil Baby Mattamana[3], Michelle Ash[1], Anjelica Reyes[1], Pavan Bhimalli[1], Neil Kelleher[3], Vinayaka Prasad[2], Jeffrey Schneider[1]

[1]*Department of Microbial Pathogens and Immunity, Rush University Medical Center, Chicago, IL,* [2]*Albert Einstein College of Medicine, Bronx, New York,* [3]*Northwestern University Proteomic Center of Excellence.,*

The central nervous system (CNS) is a viral reservoir for HIV infected individuals despite the use of combined antiretroviral therapy (cART). Following cART cessation, HIV viral egress from these sanctuary sites, such as the CNS, cause viral rebound and progression to AIDS. Immunotherapy targeting to the CNS is very inefficient, with 0.1–1% of intravenous injected antibodies reaching the brain. Utilizing archived cerebral spinal fluid and brain tissue from the National Neuro-AIDS Tissue Consortium (NNTC), we found that we could isolate immunoglobulin G (IgG) from the CNS of HIV infected individuals. We characterized the biochemical makeup of these antibodies through bulk IgG glycosylation analysis, via mass spectrometry, and found that they contain an afucosylated/asialylated skewed glycan profile when compared to matching plasma. We Cy5 conjugated these antibodies and measured their entry in an *in vitro* blood brain barrier (BBB) model containing human brain microvascular endothelial cells and astrocytes and found increased penetration for CSF-derived antibodies compared to plasma. Next, we glycoengineered VRC01 (anti-HIV broadly neutralizing antibody) using HEK293 cells with FUT8 and ST6GAL1 knocked down, to reduce fucose and sialic acid levels, respectively, in order to mimic CNS-derived IgG. We Cy5 conjugated these glycoengineered antibodies and found increased penetration in the BBB assay compared to unmodified IgG. Taken together, our results have shown the feasibility of characterizing CNS-resident antibodies and the possible exploitation of these antibodies' glycosylation patterns to increase their penetrance into the CNS. In future studies we will glyco-engineer anti-HIV antibodies in an attempt to block viral egress from the brain in a CNS-specific HIV infection/cART cessation humanized mouse model. These findings have the potential to not only aid in the HIV cure strategy but could increase efficacy of drugs targeting a myriad of neurodegenerative diseases which rely on therapeutic antibodies.

(P-084) SARS-CoV-2 Omicron subvariants maintain glycosylation sites on the spike protein, but alterations in glycosylation patterns at these sites may contribute to immune evasion

Bhoj Kumar[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA,*

SARS-CoV-2 spike protein glycosylation plays a critical role in viral infection, influencing protein structure, receptor binding, and immune evasion. Several currently circulating variants of interest (VOIs) of the Omicron sublineage have evolved during the post-pandemic, containing crucial mutations on the S protein of the virus leading to increased transmissibility and immune escape. While the glycosylation sites on the spike protein remain consistent across Omicron subvariants, our study reveals significant alterations in specific glycosylation patterns. We analyzed the N- and O-glycosylation profiles of six Omicron subvariants (XBB.1.5, XBB.1.16, EG.5, BA.2.75, BA.2.86, and XBB.2.3). Our detailed profiling of the glycosylation at each of the individual sites of the S protein across the variants revealed an association of glycosylation pattern on the variants and their infectivity. Our findings demonstrate that variants with increased transmissibility and immune escape tend to exhibit reduced levels of oligomannose glycans and elevated complex-type glycans. Notably, the most recent variants displayed the most distinct glycosylation profiles. Specific glycosylation changes were identified in both the N-terminal domain (NTD) and receptor-binding domain (RBD) of the spike protein. These results highlight the dynamic nature of SARS-CoV-2 glycosylation pattern of the SARS-CoV-2 variants and its potential contribution to infectivity. Our study underscores the importance of both N- and O-glycosylation in SARS-CoV-2 infection, as these modifications are highly conserved across all analyzed variants.

(P-085) Glycosylation Modifications in Lyme Disease: Immunoglobulins to Specific vs. Nonspecific Bacterial Antigens

Mary Ann Comunale*[,1], Aline Guadalupe Coelho[1]

[1]*Drexel University College of Medicine,*

*Corresponding Author.

Infection with *Borrelia burgdorferi (Bb)*, the causative agent of Lyme disease, does not result in disease-resolving antibody production or long-term protective immunity. It is known that increased levels of sialic acid on IgG sterically impair the Fc

region's affinity toward lymphocytic receptors such as FcγR IIIA and classical complement cascade activation via C1q. These responses are crucial to destroy the bacteria and release more bacterial antigens for the proper sequence of an adaptive immune response.

We examined the IgG glycosylation of antibodies from patients with acute Lyme disease and healthy controls. Glycosylation changes on immunoglobulins specific to *Borrelia burgdorferi* antigens differ from nonspecific bacterial proteins in the serum of acute Lyme disease patients. Furthermore, the glycosylation changes have a deleterious effect on host immune response. By examining the glycosylation of immunoglobulins produced in response to acute Lyme disease, we demonstrate a mechanism that helps to explain why patients require prompt antibiotic therapy to recover.

(P-086) Engineering bacterial oligosaccharyltransferases towards the production of novel conjugate vaccines

Kathryn A. Myers[1], Derek A. Wong[2,3,4], Zachary M. Shaver[3,4], Michael C. Jewett[1]

[1]*Department of Bioengineering, Stanford University, Stanford, CA,* [2]*Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL,* [3]*Chemistry of Life Processes Institute, Northwestern University, Evanston, IL,* [4]*Center for Synthetic Biology, Northwestern University, Evanston, IL,*

Glycoconjugate vaccines, consisting of a pathogen-specific polysaccharide antigen linked to an immunogenic carrier protein, are a powerful tool in the global fight against infectious diseases. These vaccines are currently manufactured via the non-specific chemical conjugation of an antigen isolated from fermented pathogens to a purified recombinant carrier protein. These manufacturing methods are expensive and require large-batch growth of pathogens to obtain the polysaccharide antigens. Recent work in bacterial glycoengineering has leveraged both *in vivo* and cell-free methods for producing conjugate vaccines via bacterial oligosaccharyltranfserases (OSTs), which can site-specifically transfer polysaccharide antigens to carrier proteins of interest. These methods circumvent many challenges associated with existing manufacturing platforms. Unfortunately, the limited glycan substrate scope of known OSTs for high-interest bacterial pathogens limits their implementation. Here, we highlight our efforts to screen new OSTs to expand the number of antigens of clinical relevance that can be recognized and transferred to carrier proteins. First, we demonstrate the ability to express membrane-bound OSTs in nanodisc-supplemented cell-free expression reactions. Next, we couple cell-free expression with *in vitro* glycosylation to show these OSTs can glycosylate carrier proteins with polysaccharide antigens such as those from a *Streptococcus pneumoniae* serotype. We successfully screened over 50 bacterial OSTs, some of which were shown to catalyze N- or O-linked glycosylation in enriched cell-free extracts. Finally, we propose future workflows for high-throughput design and testing of OST mutant libraries to rapidly engineer these OSTs towards the production of novel glycoconjugates vaccines. This workflow will accelerate the development of, and access to, much needed conjugate vaccines.

(P-087) Genome mining unearths new bacterial oligosaccharyltransferases for efficient biosynthesis of conjugate vaccines

Yufan Qin[1], Thomas Connor Donahue[1], Sophia W. Hubert[2], Ching-Chih Feng[1], Michael C. Jewett[3], Matthew P. DeLisa[1,2]

[1]*Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853, USA,* [2]*Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA,* [3]*Department of Bioengineering, Stanford University, Stanford, CA 94305, USA,*

Vaccination is a highly effective approach in preventing and controlling infectious diseases. Conjugate vaccines represent a specialized type of vaccine designed to combat pathogenic bacteria by targeting unique cell surface antigens. These vaccines link polysaccharides from the bacterial cell surface with a highly immunogenic carrier protein to stimulate a robust immune response in humans. Conjugates are a safe and effective strategy for protecting against diverse virulent pathogens, with successful vaccines licensed worldwide against *Haemophilus influenzae*, *Neisseria meningitidis* serogroups, *Streptococcus pneumoniae*, and *Salmonella typhi*, and other promising candidates in various stages of clinical development. Despite their effectiveness, traditional conjugate vaccines have several drawbacks. Most notably is the complex, multistep process required to purify, isolate, and conjugate bacterial polysaccharides, which is costly, time and labor intensive, and low yielding. To sidestep these issues, metabolic engineering of bacteria has emerged as an attractive alternative for one-step biosynthesis of an unlimited and renewable supply of conjugate vaccines. This bioconjugation approach leverages engineered protein glycosylation in non-pathogenic *Escherichia coli* strains that are capable of conjugating recombinantly produced polysaccharide molecules to co-expressed carrier proteins by an oligosaccharyltransferase (OST) such as PglB from *Campylobacter jejuni* (*Cj*PglB). However, the historical reliance on *Cj*PglB for glycosylation has limited the range of polysaccharides that can be efficiently transferred to carrier proteins. To address this

limitation, we have embarked on a genome mining expedition with the goal of uncovering previously uncharacterized *Cj*PglB homologs with increased catalytic efficiency towards polysaccharide antigens that only weakly transferred, if at all, by *Cj*PglB. To date, we have conducted a comprehensive screening of > 50 *Cj*PglB homologs. Each of these OSTs was evaluated for the ability to efficiently transfer different capsular and O-antigen polysaccharide antigens from *Francisella tularensis*, *Shigella flexneri*, and *Streptococcus pneumoniae*, among others, onto an FDA-approved carrier protein, namely nonacylated *Haemophilus influenzae* protein D (PD), engineered with a C-terminal glycosylation tag. Our screening process uncovered several PglB homologs that exhibit higher glycosylation efficiency of PD compared to *Cj*PglB, suggesting that bacterial OSTs other than *Cj*PglB can achieve higher glycosylation efficiency in conjugate vaccine production. The discovery of these ultra-efficient conjugating enzymes has the potential to not only unlock previously untransferable polysaccharides but also paves the way for cost-effective biomanufacturing workflows that enable development of optimally glycosylated conjugates against a range of bacterial pathogens.

(P-088) Innate Immune Galectin Protects Against Streptococcus pneumoniae infection

Hau-Ming Jan[*,1], Shang-Chuen Wu[*,1], Connie M. Arthur[1], Sean R. Stowell[1]

[1]*Joint Program in Transfusion Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA,*

Streptococcus pneumoniae is a major contributor to global mortality, causing severe infections such as meningitis and septicemia. Annually, it results in 1.6 million deaths in developing countries, with one million of these deaths occurring in children under five years old. The antibiotic-resistant serotype 14 is particularly problematic due to its broad-spectrum resistance to beta-lactams, complicating treatment protocols and increasing failure rates. Notably, the expression of mammalian-like antigens on the surface of serotype 14 leads to the deletion of cells producing self-specific antibodies, thereby limiting adaptive immunity against pathogens with similar antigens. Given these limitations in adaptive immunity, we investigated innate immune proteins in the lung previously suggested to target and eliminate pathogens that utilize molecular mimicry. Using this approach, we identified galectin-1 (Gal-1) and galectin-3 (Gal-3) as major lung lectins using LC-MS/MS analysis. Microarray analysis revealed that Gal-1 has a stronger binding affinity to serotype 14 compared to Gal-3 and other galectins. *In vitro* assays demonstrated that Gal-1 effectively kills serotype 14. Furthermore, *in vivo* experiments revealed that the removal of Gal-1 made recipients particularly susceptible to serotype 14, whereas similar reductions in Gal-3 had minimal impact. These findings suggest that Gal-1 provides an important source of host innate immunity against molecular mimicry.

(P-089) Sex-specific roles of gut epithelial ST6Gal1 in mucosal homeostasis and protection from acute and infection-induced colitis in mice

Mackenzie Melvin[1], Simin Jafaripour[1], Noah Fancy[1], Jackline Irungu[2], Pirandis Daneshgar[2], Rain Inaba[1], Geetkamal Hans[1], Caixia Ma[3], Qiaochu Liang[3], Bruce A. Vallance[3], Wesley F. Zandberg[2], Kirk S.B. Bergstrom[1]

[1]*Dept. of Biology,* [2]*Dept. of Chemistry, University of British Columbia, Okanagan Campus, Kelowna, BC, Canada,* [3]*Dept of Pediatrics, BC Children's Hospital, Vancouver, BC Canada,*

Mucin-2 (MUC2) is a gel-like polymer that comprises gut mucus and forms a protective layer in the colon. A major sugar found on MUC2 is sialic acid (Sia), which has recently been implicated in mucus functions. The sialylation of mucus can occur via different linkages, where one of the most observed is an α2,6 linkage to galactose (Gal). Importantly, α2,6-linked Sia acts as a capping structure on mucus N- and possibly O-glycans that is presented to and interacts with the gut microbiota. The addition of α2,6 Sia to these structures is mediated by beta-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal1), which is implicated in sex-specific pathologies. While the role of ST6Gal1 in modulating the immune system and its signaling pathways has been well-studied, the impact on gut mucus and physiology, and its role in microbiota modulation are unknown. Additionally, while ST6Gal1 is implicated in sex-specific pathologies, the role of sex-specific sialylation in gut mucosal function is not clear. To address these questions, a mouse line that conditionally lacks ST6Gal1 in the gut epithelium using CreLox Technology to target Siaα2,6Gal sialylation was derived. Lectin staining revealed a loss of *Sambucus nigra* agglutinin (SNA)-1 (Siaα2,6Gal-binding) signal in the colon crypts of intestinal epithelial cell (IEC) *St6gal1*$^{-/-}$ mice, indicating that ST6Gal1 is the sole contributor of Siaα2,6Gal in the colon. Baseline mucus function, colon physiology, or stem cell function (assessed via colonoid growth assays) revealed no differences between genotypes or sexes in our facility. However, there were significant sex- and genotype-specific differences when looking at short-chain fatty acid (SCFA) and microbiota profiles assessed through 16S rRNA profiling, indicating an impact of ST6Gal1 on colonic microbial ecology. Treatment with dextran sodium sulfate (DSS), an inducer of acute colitis, revealed a mildly worsened clinical score and disease overall in male vs. female IEC *St6gal1*$^{-/-}$ mice and compared to *St6gal1f/f* littermates. Further, the *Citrobacter rodentium* infection model highlighted worsened infection in the absence of ST6Gal1, with

bacterial lipopolysaccharide (LPS) staining and pathology suggesting a more widespread and heightened infection of distal and cecal tissues in female IEC *St6gal1*$^{-/-}$ mice. Collectively, this study expands the current knowledge surrounding the physiological role of ST6Gal1 in vivo and its sex-specific impact on gut homeostasis and mucus sialylation.

(P-090) Local glycan engineering induces systemic antitumor immune reactions via antigen cross-presentation

Natalia Rodrigues Mantuano[1], Michael Sandholzer[1], Andreas Zingg[1], Filip Filipsky[1], Ronja Wieboldt[1], Alfred Zippelius, Heinz Läubli[1,2]

[1]*Department of Biomedicine, University Hospital and University of Basel, Switzerland,* [2]*Division of Oncology, University Hospital Basel, Switzerland,*

The advent of immune checkpoint inhibition (ICI) has revolutionized cancer treatment, yet response rates remain modest, and mechanisms of resistance are poorly understood. Recent studies highlight the role of sialic acid-containing glycans and their interaction with sialic acid-binding immunoglobulin-like lectin (Siglec) receptors in the tumor microenvironment, offering a promising treatment option for cancer immunotherapy. In this work, we performed glyco-engineering of the tumor microenvironment by using an adeno-associated virus (AAV) constructed to express glycosidases including influenza A neuraminidase or human fucosidase A (AAV-sia or AAV-FUCA1). Upon AAV-sia treatment, cancer cells expressed sialidase on the cell surface and were able to cleave sialic acid in the tumor microenvironment in various mouse models. Furthermore, a relevant synergism combining AAV-sia and anti-PD-1 treatment was observed. In addition, combination with human fucosidase (AAV-FUCA1), sialidase and PD-1 was able to significantly increase survival and the rate of complete cure. Mechanistic studies demonstrated an increased activation of $CD8^+$ T cells, and an increase in conventional dendritic cell type 1 (cDC1) infiltration. Despite local injection and confined desialylation, we observed a growth inhibition on distant tumor sites and an increase in tumor-specific T cells suggesting a systemic immune activation. Additional studies showed that antigen cross-presentation of cDC1 was increased upon sialidase treatment, enhancing anti-tumor T cell responses. Taken together, locally delivered glycosidases to tumor microenvironment can induce systemic anti-tumor immunity by enhancing antigen cross-presentation, thereby enhancing T cell mediated cancer cell killing.

(P-091) Characterization of site-specific N-glycosylation profiles of envelope glycoproteins from alphaviruses virus-like particles (VLPs)

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,*

Alphaviruses are enveloped, positive-sense, single-stranded RNA viruses with substantial impact on animal and human health. Transmitted by mosquitos, alphavirus infection in humans result in a spectrum of disease that can be categorized as either arthritogenic or encephalitic. Multiple vaccine and therapeutic candidates have been explored to combat alphavirus infections, yet none have been approved for public use in the United States. Encephalitic alphaviruses, eastern and Venezuelan equine encephalitis virus (EEEV and VEEV), and potentially arthritogenic alphavirus, chikungunya virus (CHIKV), can be transmitted via aerosol exposure. Here, we analyzed the site-specific N-glycosylation patterns of the envelope glycoproteins from CHIKV, EEEV, and VEEV virus-like particles (VLPs). In addition to the existing endogenous N-linked glycan sites, we also engineered novel glycan sites to see if they affect the antigenicity and immunogenicity of the VLPs. Our glycoproteomic data showed that more than 90% of the sites were highly occupied (with occupancy higher than 75%), and more than 55% of the sites were occupied by an abundance of complex glycans (more than 50%). An antibody binding analysis as well as immunization analysis was also performed and the results illustrated a site-specific correlation of both glycosylation occupancy and type with antigenicity. These initial findings are currently being utilized to facilitate the production of selective antibodies that target specific glycan sites on the VLPs with the long-term goal of generating effective broadly neutralizing antibodies and vaccines against alphaviruses.

(P-092) Comparison of N- and O-glycosylation on Spike Glycoprotein 1 of SARS-CoV-1 and MERS-CoV

Yuan Tian[1], John F. Cipollo[1]

[1]*Food and Drug Administration, Center for Biologics Evaluation and Research, Division of Bacterial, Parasitic and Allergenic Products, Silver Spring, Maryland, USA,*

Coronaviruses (CoVs) are enveloped pathogens causing multiple respiratory disorders in humans with varying severity. Spike protein is one of the major proteins expressed on coronavirus surface, which mediates coronavirus entry into host cells. Spike proteins are extensively glycosylated and the glycans displayed on spike proteins play a key role in host pathogenesis and immune evasion. In this study, we aim to investigate whether glycosylation patterns are conservative at certain glycosites across different coronaviruses and how different host cells impact on the glycosylation profile. We analyzed site-specific glycans of S1 subunit from SARS-CoV and MERS-CoV spike proteins using hydrophilic interaction chromatography (HILIC) and LC-MS/MS on an Orbitrap Eclipse Tribrid mass spectrometer. We also compared glycosylation of MERS-CoV spike protein derived from HEK293 and insect cells. Our results show that the *N*-glycosylation has some common pattern in SARS-CoV S1 and MERS-CoV S1 and also reveal the similar locations of *O*-glycosites identified in the two coronaviruses.

(P-093) Identifying and characterizing key determinants of glycoantigen-MHCII interactions

Trevor Adams[1], Sena Cinar[1], Fikri Avci[1]

[1]*Emory University,*

A major step in the activation of CD4+ helper T cells is the presentation of antigens to T cell receptors (TCRs) via major histocompatibility complex II (MHCII). Currently, the role of peptide glycosylation in MHCII binding is not well understood. This is of particular interest in finding novel epitopes for densely glycosylated viral glycoproteins, such as the Env glycoprotein found in HIV. In previous work, our group identified a glycosylated epitope that was capable of being presented to a TCR and stimulating an immune response. Here, we seek to understand the key determinants and propensities of glycopeptide binding to MHCII molecules using *in vitro* approaches. We expressed three variants of HIV-Env in Exp293 cells as native flexibly linked (NFL) trimers that represent three major clades of virus: A (BG505), B (JR-FL), and C (16055). The site-specific glycosylation of these trimers was characterized using glycomics and glycopeptide analysis. The interactions between candidate glycopeptides and MHCII are probed using *in silico* molecular dynamics simulations. We are establishing a cell-free antigen processing and presentation system to identify new glycopeptide epitopes that are capable of binding MHCII, and thus may play a role in stimulating an immune response. This work furthers our understanding of how glycoantigens are processed and presented to the immune system, and has potential applications in the development of novel vaccines and therapeutics.

(P-094) The Role of Glycosylation in Modulating ACE2- SARS-CoV-2 Spike Binding Affinity and Anti-Spike Monoclonal Antibody Recognition

Paige LaMore[1], Eliza Gazaway[1], Oliver C. Grant[1], Jeremy Praissman[1], Digantkumar Chapla[1], Annapoorani Ramiah[1], Kelley Moremen[1], Lance Wells[1], Robert J. Woods[1]

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

Severe acute respiratory coronavirus 2 (SARS-CoV-2) is a highly contagious virus that results in coronavirus disease (COVID) with symptoms that range from mild flu-like to death. A key step in SARS-CoV-2 pathogenesis is the adhesion of Spike glycoprotein to the host cell receptor angiotensin-converting-enzyme 2 (ACE2). As a result of selective pressures, SARS-CoV-2 S has evolved enhanced receptor binding and decreased antibody recognition via point mutations and glycosylation contributing to overall increased infectivity. However, glycosylation of viral antigens and host receptor proteins can also impact their binding. Previous studies revealed that the Spike trimer adopts a conformational change to an open state to expose its receptor binding domain (RBD) for ACE2 binding. Additionally, ACE2 has glycans located near the RBD of Spike, suggesting that ACE2 glycans play a role in mediating Spike binding. Our studies reveal three specific ACE2 glycans that interact with the RBD and nearby glycans of Spike. Furthermore, transcriptomic studies show that two of the three glycosites are alleles in humans that result in loss of site-specific glycosylation on ACE2. However, it is not well understood how Spike glycosylation affects the antigenicity of both human and therapeutic antibodies. Our structural analyses indicate that ACE2 and mAbs have overlapping interaction

sites on the Spike RBD variants of concern (VOC). Therefore, we propose that **glycosylation on ACE2 and trimeric Spike of SARS-CoV-2 regulates effective mAb binding, and therefore, a deeper understanding of this relationship will aid in the design of novel therapeutic mAbs to inhibit viral infection.** Here, we utilize biolayer interferometry to quantify the impact of variations of ACE2 and Spike glycosylation in addition to VOC on anti-Spike mAb binding affinity in terms of K_D. Additionally, we perform molecular dynamic simulations and antigenic surface analyses to elucidate the structural features of glycans on ACE2 and Spike VOC mutations that contribute to conformational changes that mediate anti-Spike mAb binding. These experiments will reveal how the glycosylation on ACE2 and Spike mediate viral infection and immune evasion in emerging strains of SARS-CoV-2. Furthermore, this research will provide insights into differences in viral infectivity and treatment effectivity seen within the human population.

(P-095) Siglec-7 is a glyco-immune checkpoint regulating distinct human T cell niches

Lukas Muerner[1], Aref Hosseini[1], Jovana Jankovic[1], Gennaro de Libero[2], Richard D. Cummings[3], Stephan von Gunten[1]

[1]Institute of Pharmacology, University of Bern, 3010 Bern, Switzerland, [2]Experimental Immunology, Department of Biomedicine, University Hospital Basel, University of Basel, Basel, Switzerland, [3]Department of Surgery Beth Israel Deaconess Medical Center, National Center for Functional Glycomics, Harvard Medical School, Boston, MA, 02215, U.S.A.,

Sialic acid-binding immunoglobulin-like lectin 7 (Siglec-7) is a sialoglycan-specific inhibitory receptor differentially expressed on leukocytes. While Siglec-7 is only expressed on a subset of peripheral blood T lymphocytes in healthy donors, recent work of our group showed that Siglec-7 is significantly upregulated on tumor-infiltrating lymphocytes (TILs) in melanoma and acute myeloid leukemia (AML). However, up to this date, the regulatory mechanisms underlying the cell-type- and subset-specific expression of *SIGLEC7*, as well as the role of Siglec-7 in T cell biology remain unexplored. To extend previous characterization efforts of Siglec-7+ CD8+ T cells, we performed in-depth phenotyping of Siglec-7 positive and negative T cell subsets using RNA-sequencing and flow cytometry. Transcriptome analysis suggests enriched expression of *SIGLEC7* in distinct T cell populations which revealed characteristic gene expression signatures. These findings were corroborated by flow cytometric analysis of respective lineage markers (chemokine receptors, transcription factors, etc.), as well as T cell receptor (TCR) analysis. Moreover, functional assays revealed proliferation and expression of characteristic cytokines, as a response to specific TCR and/or cytokine stimulation. Furthermore, analysis of chromatin-immunoprecipitation sequencing (ChIP-seq) datasets and luciferase-reporter assays identified candidate cis-regulatory elements and transcription factors involved in the regulation of subset-specific Siglec-7 expression. These findings were corroborated by the analysis of CITE-Seq datasets suggesting expression of Siglec-7 in specific T cell clusters in diverse human tissues. Finally, we studied the Siglec-7 interactome using proximity labeling and candidate interactors were verified using gene knockout studies in primary human T cell clones. Yet, the role of Siglec-7 in the regulation T cell populations, the context of Siglec-7 induction and ligand interaction needs to be further investigated. Given the need for novel immunotherapies for cancer and autoimmune diseases, and considering the abundant expression of sialoglycans on tumor as well as healthy tissues, exploiting this sialoglycan-specific immune checkpoint could be a promising therapeutic strategy.

(P-096) The GPI Sidechain of Toxoplasma gondii Inhibits Parasite Pathogenesis

Julia A. Alvarez[1], Elisabet Gas-Pascual[2], Sahil Malhi[1], Juan C. Sánchez-Arcila[1], Ferdinand Ngale Njume[1], Hanke van der Wel[2], Yanlin Zhao[1], Laura García-López[1], Gabriella Ceron[1], Jasmine Posada[1], Scott P. Souza[1], George S. Yap[3], Kirk D.C. Jensen[1], Christopher M. West[2]

[1]Dept. of Molecular and Cell Biology, School of Natural Sciences, Quantitative and Systems Biology Graduate Program, and Health Sciences Research Institute, University of California, Merced CA, [2]Dept. of Biochemistry and Molecular Biology, Center for Tropical and Emerging Global Diseases, and Complex Carbohydrate Research Center, University of Georgia, Athens GA, [3]Dept. of Medicine and Center for Immunity and Inflammation, New Jersey Medical School, Rutgers University, Newark NJ,

Glycosylphosphatidylinositols (GPIs) are highly conserved free glycolipids and anchors for eukaryotic cell surface proteins. While the glycan backbone is conserved, species differ in sidechains added to the triple mannose core. The functional significance of sidechain modifications to the GPI anchor in parasites is yet to be determined because the responsible glycosyltransferases have not been identified. The apicomplexan *Toxoplasma gondii* is a widespread intracellular pathogen of warm-blooded animals whose plasma membrane is covered with GPI-anchored proteins and free GPIs called GIPLs. The functional significance of the Glcα1,4GalNAcβ1- sidechain reported in *T. gondii* has remained largely unknown without an understanding of its biosynthesis. Here we identify and disrupt two glycosyltransferase genes and confirm their respective roles by serology and mass spectrometry. Informatic anlysis and immunolocalization studies suggest that the βGalNAcT, termed PIGJ, encodes a type 2 transmembrane protein with a rER luminal glycosyltransferase domain with greatest similarity to CAZy family GT17, and its putative catalytic DxD motif suggests it to be UDP-GalNAc dependent. The αGlcT, named PIGE, is also predicted

to be a type 2 transmembrane protein with greatest similarity to CAZy GT32 glycosyltransferases, and its DxD-like motif is consistent with reported UDP-Glc dependence of the enzyme activity in extracts. Parasites lacking the sidechain on account of deletion of the first glycosyltransferase, PIGJ, exhibit increased virulence during primary and secondary infections, suggesting it is an important pathogenesis factor. Cytokine responses, antibody recognition of GPI-anchored SAGs (Surface AntiGens), and complement binding to *pigj*-KO mutants are intact. In contrast, the scavenger receptor CD36 shows enhanced binding to *pigj*-KO parasites, potentially explaining a subtle tropism for macrophages detected early in infection. Galectin-3, which bind GIPLs, exhibits an enhancement of binding to *pigj*-KOs, and the protection of galectin-3 knockout mice from lethality suggests that *pigj*-KO parasite virulence in this context is sidechain dependent. Parasite numbers are not affected by deletion of *pigj* early in the infection in wildtype mice, suggesting a breakdown of tolerance. However, increased tissue cysts in the brains of mice infected with *pigj*-KO parasites indicate an advantage over wildtype strains. Thus, the GPI disaccharide sidechain of *T. gondii* plays crucial and diverse rols in regulating disease outcome in the infected host.

(P-097) HIV-1 p17 Interactions with Heparan Sulfate

Adrian Ortiz-Arevalo[1], Brandon Chavez[1], Rochelle Johnson[1], Kenia Vidal[1], Stephanie Rauda[1], Bradley Heath[2], Alexander W. Sorum[3], Linda C. Hsieh-Wilson[3], Kari Pederson[1]

[1]Department of Chemistry & Biochemistry, California State University, Dominguez Hills, Carson, CA 90747, [2]Clinical Sciences Program, California State University, Dominguez Hills, Carson, CA 90747, [3]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125,

Glycosaminoglycans (GAGs) are expressed ubiquitously on mammalian cell surfaces and interact with a wide variety of biological molecules to modulate processes, including immune response, regulation of cell growth and blood-stream clotting. Disruption of GAG-binding has the potential to prevent infection by viruses, such as HIV-1, and to reduce inflammation caused by autoimmune disorders. Although treatments to prevent the HIV-1 virus from cell entry are not yet available, the target of this study, p17, is a structural protein that may present a target for this type of treatment as it involved in a majority of the stages of the life cycle of the virus, including cell adhesion and viral entry through interactions with heparan sulfate proteoglycans (HSPG). Unfortunately, the diversity of potential binding motifs in GAG sequences has made identification of relevant sequences challenging. In particular, heparan sulfate (HS) is the most structurally diverse, with sixteen unique disaccharide configurations and 256 unique tetrasaccharide configurations. Significant progress has been made in recent years developing libraries of synthetic HS oligomers, allowing for detailed experimental structural studies of HS-protein complexes.

Here we examine the sequence-specificity of the heparan sulfate-p17 interaction using microarray screening and NMR titration. HIV-1 p17 was screened against a microarray of 64 synthetic heparan sulfate tetrasaccharides and preferential binding was observed for 2-O-sulfated heparan sulfate tetrasaccharides. A set of heparan sulfate tetrasaccharides including strong, medium, and weak binding ligands were selected for NMR titration studies. Protein residues that interact with each of the tetrasaccharides were identified. Additional STD and transferred-NOESY experiments will be used to identify structural features needed for strong and specific binding and the protein-ligand complexes will be modeled computationally. Understanding the interactions between HIV-1 p17 and heparan sulfate could be a key factor in the development of potential antiviral drugs.

(P-098) Binding of hypersensitivity-associated human IgE antibodies to non-human glycans in the Fc region of a model monoclonal antibody biotherapeutic

Grayson Hatfield[1], Lioudmila Tepliakova[1], Jessica Tran[1], Huixin Lu[1], Michel Gilbert[2], Roger Y. Tam[1]

[1]Cntre for Oncology, Radiopharmaceuticals and Research, Biologic and Radiopharmaceutical Drugs Directorate, Health Canada, [2]Human Health Therapeutics Research Centre, National Research Council Canada,

Monoclonal antibodies (mAbs) are biologic therapeutics that are used to treat cancer, chronic inflammation, and protect against respiratory infections. Glycosylation fo these glycoprotein-based biologics influence mAb function, potency and safety. Their production in non-human cells can incorporate non-human glycan structures including galactosyl-α1-3-galactose (α1-3-Gal) groups. Cetuximab is a commercial therapeutic mAb associated with causing anaphylaxis in some patients due to endogenous anti-α1-3-Gal IgE binding, which was found to occur in the 'Fab' but not the 'Fc' region of the mAb molecule. Although previous literature did not detect any binding to the Fc region, the heterogenous nature of glycans in the commercial products used in those studies limited their detailed examination. Despite their low abundance in the Fc region in typical commercial mAbs, the sensitivity of manufacturing conditions on glycosylation profiles and the development of novel glycoengineering strategies, antibody-based modalities and biosimilars by various manufacturers necessitates a better understanding of the structural requirements for anti-α1-3-Gal IgE binding to the Fc region. We synthesized mAbs derived from a commercially-available therapeutic, where various chemically-defined glycans comprising non-human α1-3-Gal groups were incorporated.

Using these mAb analogues with homogeneous glycans, we tested their relative binding to two commercial anti-α1-3-Gal human IgE antibodies derived from a patient with allergies to red meat (comprising α1-3-Gal epitopes), and to the FcγRIIIA receptor that involved in antibody-dependent cellular cytotoxicity. While previous literature reported α1-3-Gal glycans in the Fc region do not bind to anti-α1-3-Gal human IgE, our results show that by using mAbs with chemically-defined and homogeneous glycans, anti-α1-3-Gal human IgE antibodies can indeed bind, with bivalent α1-3-Gal groups being the most important factor. Monovalent α1-3-Gal glycans bound less and was only slightly higher than negative controls. A detailed understanding of how non-human glycans affect the safety/potency of antibody-based biotherapeutics is important for both manufacturers and Regulatory Reviewers. Our results showing that the presence of bivalent α-Gal glycans in the Fc region can bind to anti-α-Gal human IgE suggests their consideration as a potential critical quality attribute, particularly in novel mAb-based biotherapeutic platforms. Future works include further validating these findings using *in vitro* cellular models.

Session 3: Roles for Intracellular Glycosylation

(Key3-001) Roles of O-GlcNAcylation in Ribogenesis, and in Regulating Translation and Proteostasis

Gerald W. Hart[1], Jie L. Tian[1], Quira Zeidan[2], Junfeng Ma[3], Farzad Eslami[1], Lance Wells[1]

[1]*Complex Carbohydrate Research Center, Department of Biochemistry and Molecular Biology, University of Georgia,* [2]*Present address: FIRE: The First-Year Innovation & Research Experience Program, The University of Maryland,* [3]*Present address: Lombardi Comprehensive Cancer Center, Georgetown University Medical Center.,*

Since its discovery over four decades ago, O-GlcNAcylation has emerged as a major nutrient sensor that modifies more than eight thousand human proteins to regulate nearly every cellular process, including signaling, transcription, translation, cell division, mitochondrial function, and the cytoskeleton. Over five thousand papers from many laboratories have described O-GlcNAc's roles in many cellular functions and pathways. O-GlcNAc modifies over eighty percent of human kinases, it regulates the flow of genetic information at nearly every level and is a major mechanism of nutrient regulation of transcription. As a major nutrient sensing regulator, O-GlcNAcylation plays a fundamental role in the etiology of diseases of aging, including diabetes, neurodegeneration, cancer, and cardiovascular disease.

Recently, we have investigated the roles of O-GlcNAcylation in translation and proteostasis. While the O-GlcNAc transferase (OGT) is mostly nuclear, the enzyme is completely excluded from the nucleolus (the site of ribogenesis). In contrast, O-GlcNAcase is mostly cytoplasmic but is enriched within the nucleolus. Overexpression of OGT, which 'leaks' into the nucleolus, causes severe disruption of nucleolus organization. OGT and OGA are tightly bound to the ribosome. Upon inhibition of the proteasome, both enzymes rapidly increase on ribosomes and O-GlcNAcylation (OGN) of ribosome associated proteins also increases, concomitant with inhibition of protein synthesis. Protein synthesis recovers concomitant with reduced OGN on ribosome proteins. There is extensive crosstalk between O-GlcNAcylation and ubiquitination. Knockdown of OGT reduces ubiquitination and over-expression of OGT increases ubiquitination. MS/MS analyses show that 74/82 ribosome subunits, numerous translation factors, ribosome quality control proteins, proteasome subunits, and RNA splicing factors are O-GlcNAcylated. The extent of O-GlcNAcylation of many of these proteins correlates with proteasome inhibition. These studies establish the foundation for mechanistic studies into how nutrients regulate protein translation and communication between the proteasome and the ribosome.

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(Key3-002) O-GlcNAc regulation beyond the enzyme active site

Connor M. Blankenship[1], Jinshan Xie[1], Caroline Benz[2], Ao Wang[1], Ylva Ivarsson[2], Jiaoyang Jiang[1,*]

[1]*Pharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA,* [2]*Department of Chemistry – BMC, Uppsala University, Uppsala, Sweden,*

The modification of intracellular proteins with O-linked β-*N*-acetylglucosamine (O-GlcNAc) moieties is a highly dynamic process that spatiotemporally regulates nearly every important cellular program. Despite its significance, little is known about the substrate recognition and regulation modes of O-GlcNAc transferase (OGT), the primary enzyme responsible for O-GlcNAc addition. In this study, we have identified the intervening domain (Int-D), a poorly understood protein fold found only in metazoan OGTs, as a specific regulator of OGT protein-protein interactions and substrate modification. Utilizing an innovative proteomic peptide phage display (ProP-PD) coupled with structural, biochemical, and cellular characterizations, we discovered a novel peptide motif, employed by the Int-D to facilitate specific O-GlcNAcylation. We further show that disruption of Int-D

binding dysregulates important cellular programs including nutrient stress response and glucose metabolism. These findings illustrate a novel mode of OGT substrate recognition and offer the first insights into the biological roles of this unique domain.

(Key3-003) O-GlcNAcylation: Mitochondria's Sweet Tooth for Metabolic Plasticity in Neurons

Gulcin Pekkurnaz[1]

[1]*University of California San Diego,*

Neurons require efficient metabolic processes to meet their high energy demands, especially during synaptic activity. The spatial organization of these processes within the cell, known as molecular compartmentalization, optimizes energy production and utilization. The Pekkurnaz Lab studies the metabolic "geography" within neurons, focusing on how the spatial organization of enzymes and metabolites contributes to metabolic efficiency and homeostasis. We investigate the role of metabolic sensing pathways and how they transmit nutrient availability information to subcellular compartments, matching energy demand with supply based on nutrient availability. Specifically, we focus on the metabolism-responsive post-translational modification O-GlcNAcylation in regulating neuronal glycolysis and mitochondrial activity. Our findings reveal that O-GlcNAcylation of the glycolytic enzyme Hexokinase 1 promotes glycosome formation on mitochondria, thereby facilitating glucose metabolism and ATP production. This modification is closely tied to neuronal activity, driving the upregulation of mitochondrial O-GlcNAcylation and promoting ATP synthesis based on fuel availability. Thus, it allows neurons to meet the energy demands of synaptic activity. To elucidate the molecular details of this regulation, we mapped the mitochondrial O-GlcNAcome, identifying key proteins that are compartmentalized within the neuron to optimize metabolic efficiency. Our research shows that disruptions in O-GlcNAcylation dynamics can lead to an inability to meet the metabolic demands of neuronal activity, highlighting the importance of precise metabolic regulation in maintaining neuronal health. By examining the molecular geography of neuronal metabolism and crossing boundaries between cell biology, molecular biology, and neurophysiology, we offer comprehensive insights into the mechanisms underlying neuronal energy homeostasis.

(Key3-004) Metabolic channeling of signaling monosaccharides in the brain....and more

Matthew S. Gentry[1,2], Craig W. Vander Kooi[1,2], Pankaj Singh[1], Matthieu Colpaert[1], Alex Cantrell[1], Tiantian Chen[1], Juan Pascual[3], Roberto Ribas[1,2], Ramon C. Sun[1,2]

[1]*Department of Biochemistry & Molecular Biology, U. of Florida College of Medicine,* [2]*Center for Advanced Spatial Biomolecule Research (CASBR), U. of Florida College of Medicine,* [3]*Department of Neurology, Weill Cornell Medical School,*

Glycosylation defects are a hallmark of many nervous system diseases. However, the molecular and metabolic basis for this pathology is not fully understood. We recently demonstrated that N-linked protein glycosylation in the brain is critically and directly connected to glycogenolysis. The primary function of glycogenolysis is to liberate glucose-1-phosphate from glycogen. We demonstrated that in addition to glucose, glucosamine is an abundant covalent constituent of brain glycogen. We defined the biosynthetic incorporation of glucosamine into glycogen by glycogen synthase and glucosamine release by glycogen phosphorylase in vitro by biochemical and structural methodologies, in situ in primary astrocytes, and in vivo by isotopic tracing and mass spectrometry imaging. We established that glycogen-derived glucosamine is a significant source of amino sugars required for protein glycosylation in the brain. Further, we demonstrated that disruption of cerebral glycogen metabolism causes global decreases in free UDP-N-acetyl-glucosamine and N-linked protein glycosylation using two glycogen storage disease models. These findings reveal a previously unknown yet key fundamental biological role for brain glycogen in protein glycosylation that has direct relevance to multiple human diseases of the central nervous system. Unpublished data will be presented regarding brain monosaccharide metabolism in multiple brain-centric diseases from both mouse models and patient samples.

(PT-017) Dynamic O-GlcNAcylation on the disordered domain of Sec24D regulates COPII function and is required for collagen transport

Tetsuya Hirata[1], Dharmendra Choudhary[2], Brittany J. Bisnett[1], Erik J. Soderblom[3], Ela Knapik[2], Michael Boyce[1]

[1]*Department of Biochemistry, Duke University School of Medicine,* [2]*Departments of Medicine and Cell and Developmental Biology, Vanderbilt University Medical Center,* [3]*Proteomics and Metabolomics Shared Resource, Duke University School of Medicine,*

Protein transport from the endoplasmic reticulum (ER) to the ER-Golgi intermediate compartment (ERGIC) or the Golgi is achieved by coat protein II (COPII)-coated vesicles. The COPII coat is comprised of inner and outer coat protein complexes, composed of Sec23-Sec24 heterodimers and Sec13-Sec31 heterotetramers, respectively, and assembled at ER exit sites (ERESs). The molecular mechanisms of how COPII-dependent transport is dynamically regulated upon various stimuli remain to be elucidated. O-GlcNAcylation, a reversible form of glycosylation found on more than 3,000 nuclear and cytosolic proteins in many organisms, is a regulator of COPII function and is found on various COPII proteins. Recently, we identified multiple O-GlcNAcylated sites on Sec24D, a vertebrate Sec24 paralog involved in collagen transport. Herein, we aimed to uncover the functional consequences of O-GlcNAcylation on Sec24D. To this end, we focused on two glycosites, T9 and S13, both of which are located on the disordered domain of Sec24D, and created knock-in (KI) cell lines in which endogenous Sec24D is tagged with myc-6x-His epitope tags together with an alanine substitution in one of the glycosites. Synchronous collagen transport experiments in KI cells and *in vivo* collagen staining in a Sec24D-deficient zebrafish model, *bulldog*, revealed that both T9 and S13 are critical to collagen transport from the ER to the Golgi. Moreover, O-GlcNAc levels of T9 and S13 on Sec24D were increased upon collagen transport. To clarify the underlying molecular mechanisms, interactomes of Sec24D WT, T9A, and S13A mutants were determined by mass spectrometry. We found numerous known and novel interactors of Sec24D and validated them individually via co-IP experiments. By knocking down Myoferlin, one of the novel hits, we uncovered the requirement of Myoferlin for collagen transport. Taken together, we found that dynamic O-GlcNAcylation in the disordered domain on Sec24D regulates collagen transport by modulating COPII assembly and interactions with other proteins, such as Myoferlin.

(PT-019) Regulation of the pro-oncogenic enzyme Fatty Acid SyNthase (FASN) by the nutrient-dependent modification O-GlcNAcylation in cancer cells

Dimitri Vanauberg[1], Céline Schulz[1], Fabrice Bray[1], Guillaume Brysbaert[1], Yannick Rossez[1], Tony Lefebvre[1]

[1]*Unit of Structural and Functional Glycobiology (UGSF), University of Lille, France,*

Fatty Acid SyNthase (FASN) is a ubiquitous enzyme working in the form of a homodimer. FASN is mainly cytosolic but it can also be associated to lipid rafts. FASN produces fatty acids using acetyl-CoA, malonyl-CoA and NAPDH, H^+. These fatty acids can be stored after esterification in the form of lipid droplets for energy storage but can also be used to synthesize second messengers, for protein palmitoylation and membranes building. FASN is therefore involved in cell proliferation and is regulated by several post-translational modifications including protein *O*-β-N-acetyl-D-glucosaminylation or O-GlcNAcylation. This modification consists of the addition of a single N-acetyl-D-glucosamine or GlcNAc residue from UDP-GlcNAc on the hydroxyl group of serine and threonine by the *O*-GlcNAc transferase (OGT). *O*-GlcNAcylation is a nutrition-dependent modification since the levels of the nucleotide-sugar UDP-GlcNAc, the donor of the GlcNAc group, depends on glucose, fatty acids, amino acids and nucleotides metabolisms.

My team previously demonstrated that FASN and OGT interact in the liver. FASN *O*-GlcNAcylation in mice livers and in HepG2 hepatic cancer cells promotes its interaction with the ubiquitinase USP2a (Ubiquitin Specific Peptidase 2a). Thus, FASN ubiquitination is reduced, what decreases its proteasomal degradation, increases its stability and activity. In addition, FASN inhibition reduces OGT protein amount and conversely OGT inhibition reduces FASN protein amount, disturbing cell cycle progression and reducing cancer cells viability. Indeed, at the pathological point of view FASN is overexpressed in many cancers and thus contributes to many hallmarks of cancer (Vanauberg *et al*., 2023). Because of its pro-oncogenic character, the study of FASN *O*-GlcNAcylation in cancer is of particular interest. First, we identified 29 *O*-GlcNAcylated sites along the enzyme and mutated some of them to investigate their role on FASN regulation. We observed in Hep3B (a hepatic cancer cell line) that *O*-GlcNAcylation at T980 is pivotal for FASN homodimerization, subcellular location and stability. At the cellular level we explore the effect of FASN mutation on cell viability, proliferation, migration and on tumor growth. We noticed that the FASN mutant T980A could promote HeLa cancer cells survival and migration but further experiments are still required to decipher the precise role of this *O*-GlcNAc site on FASN regulation.

The global aim of my project is to contemplate new therapeutic strategies focusing on FASN and OGT interaction. To do so, we studied this interaction by the use of FASN deletants. We identified FASN N-terminus as the prior interaction region with OGT, this region seems also the most *O*-GlcNAcylated part of the enzyme. On the other hand, we aim at determining which OGT domain(s) interact with FASN. This could help to identify OGT-to-FASN interaction inhibitors in the quest of a new therapeutic avenue for patients suffering cancer.

(PT-021) Regulation of Placental Growth Hormone Expression by O-GlcNAcylation

Lilyanna C. Massman[1], Yiwen Cui[2], Stephanie Olivier-Van Stichelen[1,2]

[1]*Medical College of Wisconsin, Department of Biochemistry,* [2]*Medical College of Wisconsin, Department of Obstetrics and Gynecology,*

The placenta is a temporary endocrine organ that supports fetal growth by altering maternal metabolism. Throughout a healthy pregnancy, hormones secreted by the placenta enter maternal circulation and shift metabolism towards a state of physiological insulin resistance. This naturally results in an increased supply of glucose available to cross the placenta and encourage fetal growth. Thus, regulation of placental hormones must remain tightly controlled to maintain pregnancy and ensure the health of both mother and fetus. Indeed, when placental hormone expression becomes deregulated, it can lead to metabolic diseases such as gestational diabetes mellitus (GDM). Placental growth hormone (PGH) has been shown to contribute to insulin resistance observed during pregnancy. Interestingly PGH production is negatively regulated by maternal plasma glucose levels.

Also regulated by maternal blood glucose concentration, *O*-GlcNAcylation is a key intracellular post-translational signaling molecule that modifies over 9,000 human proteins. Here, we explore how *O*-GlcNAcylation regulates PGH production during pregnancy in response to the maternal nutritional environment. First, we demonstrated that glucose-sensitive PGH expression is dependent on *O*-GlcNAcylation. Then, through analysis of primary placental cells, we showed that the X-linked enzyme *O*-GlcNAc transferase and the opposing enzyme *O*-GlcNAcase are down-regulated in male GDM placentas. Additionally, we identified Metastasis Associated Protein 1 as a candidate transcription factor that can act on the placental growth hormone promoter in an *O*-GlcNAc-dependent manner. Through continued investigation into the regulation of placental hormone expression, we hope to identify new avenues for detecting and treating metabolic diseases of pregnancy.

(PT-023) Sweet support: O-fucosylation detected by new antisera in protists promotes stable expression of a nucleocytoplasmic protein in Toxoplasma gondii

Megna Tiwari[1,2,3], Jen Teal[1], Elisabet Gas Pascual[1,2,3], Msano Mandalasi[1,2,3], Manish Goyal[5], Marla Popov[2], Ron Orlando[2,3], Neil E. Olszewski[4], John Samuelson[5], Christopher M. West[1,2,3]

[1]Center for Tropical and Emerging Global Diseases, [2]Complex Carbohydrate Research Center, [3]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, [4]Department of Plant and Microbial Biology, University of Minnesota, Twin Cities, [5]Department of Molecular & Cell Biology, Boston University School of Medicine,

Toxoplasma gondii infects about 30% of the human population worldwide and is responsible for the disease Toxoplasmosis. The parasite must adapt to various environments as it migrates through different tissues and differentiates in its definitive and alternate hosts. These adaptations require tight regulation of gene expression, protein levels, and protein activity. Of interest here is the O-fucosyltransferase (OFT) TgSPY, named after the *Arabidopsis* OFT SPINDLY, that modifies Ser/Thr residues of at least 33 nucleocytoplasmic proteins of *Toxoplasma* with a single fucose residue. In the protist world, OFT-like genes also occur in the parasites *Cryptosporidium and Acanthamoeba* and social amoeba *Dictyostelium*. OFT is highly homologous to the OGT that mediates O-GlcNAcylation of nucleocytoplasmic proteins in animals and plants, which has been implicated in mediating stress and nutritional responses. OFT is required for optimal growth of *Toxoplasma* and *Dictyostelium in vitro*, and by analogy with OGT, OFT may also mediate responses to stress. Fluorescence microscopy using the fucose-specific *Aleuria aurantia* lectin (AAL) indicates a high density of O-Fuc proteins near the nuclear pore complex (NPC) of *Toxoplasma*, which is bereft of other forms of fucosylation, To facilitate investigation of O-Fuc in fucose-rich organisms, we developed antibodies specific for fucose-O-Ser and fucose-O-Thr (anti-FOS/T). Unlike the lectin AAL that reacts with all terminal fucose residues in the secretory pathway, anti-FOS/T only detects nucleocytoplasmic proteins modified by OFT. Anti-FOS and anti-FOT label *Toxoplasma*, *Acanthamoeba*, and *Dictyostelium* nuclei in a pattern reminiscent of O-GlcNAc in animal cells. Western blotting confirms its utility in *Arabidopsis*, and shows that the *Dictyostelium* O-fucome is highly induced during starvation-induced development to form slugs and fruiting bodies. Analysis of anti-FOS and anti-FOT pull-downs from *Dictyostelium* in a mass spectrometry-based proteomics workflow yielded an expanded O-fucome compared to AAL analysis of a cytosolic fraction. Relative to OFT-KO cells, anti-FOS and anti-FOT captured partially overlapping sets of proteins that correlated with the presence of Ser-rich and Thr-rich sequences. Separate partially overlapping sets of proteins were captured from developing slugs, indicating developmental regulation. The *Dictyostelium* O-fucomes partially overlap with the O-fucomes of *Toxoplasma* and *Arabidopsis,* including proteins associated with transcription, mRNA processing, and the nuclear pore complex. By epitope-tagging a *Toxoplasma* GPN-GTPase gene, we confirmed a high level of O-fucosylation in its Ser-rich domain, and that O-fucosylation is required to achieve normal expression levels. We speculate that protist O-Fuc is responsive to external signals such as nutrition, which may control the availability of GDP-Fuc required by OFT to modulate the stability of key proteins with disordered Ser- or Thr-rich domains.

(P-099) The molecular mechanism of determining the fate of glycoproteins in the endoplasmic reticulum

Satoshi Ninagawa[1,2], Kazutoshi Mori[3]

[1]Biosignal Research Center, Kobe University, Japan, [2]Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, Japan, [3]Institute for Advanced Science, Kyoto University, Kyoto 606-8501, Japan,

How ER glycoproteins are destined for folding or degradation remains one of the biggest questions in the field of protein quality control in the endoplasmic reticulum (ER). In protein quality control in the ER, the structure of N-glycan plays pivotal roles. The N-glycan added to nascent proteins is composed of three glucoses, nine mannoses, and two N-acetylglucosamines (GlcNAc), and is termed $Glc_3Man_9GlcNAc_2$ (G3M9), which is processed to GM9 by the actions of glucosidase I and glucosidase II. Calnexin (CNX) and calreticulin (CRT) recognize glycoproteins with GM9 to facilitate productive folding. When glycoproteins with M9 attain their tertiary structure, they move on to the next compartment of the secretory pathway. If the protein portion does not form a proper structure, UDP-glucose glycoprotein glucosyltransferases (UGGTs: UGGT1 and UGGT2) re-add glucose to the glycoprotein for recognition by CNX/CRT to facilitate protein folding. If glycoproteins with M9 are not folded correctly within a certain time period, mannose residues are trimmed. Glycoproteins with a well-trimmed N-glycan are recognized by lectin degradation factors to be degraded by the proteasome in the cytosol. The series of these processes is collectively referred to as glycoprotein ER-associated degradation (gpERAD). We previously demonstrated that nine mannoses (M9) of the N-linked glycan of structurally aberrant glycoproteins are trimmed to M8B by the EDEM2-S-S-TXNDC11 complex, and that EDEM1 and mainly EDEM3 contribute to the trimming from M8B (Ninagawa et al., 2014, 2015 JCB; George∗, Ninagawa∗ et al., 2020, 2021 eLife ∗ Co-1st). We proposed this as a novel model of mannose trimming of N-glycan in the ER. UGGT has been shown to contribute to glycoprotein folding through their re-glucosylation activity, but seems not to affect ERAD, because it was previously reported that the presence of one glucose in the A branch of N-glycans did not change the timing of substrate degradation. However, we consider that it has not been directly examined whether UGGT-mediated re-glucosylation of N-glycan is involved in degradation of glycoproteins in the ER. We generated UGGT1-knockout (KO), UGGT2-KO and UGGT-double KO (DKO) cell lines to investigate this. Surprisingly, we found that degradation of misfolded and unstable glycoproteins was markedly accelerated in UGGT1-KO and DKO cells (Ninagawa et al., 2023 eLife).

Based on our experience studying the enzymes involved in folding and degradation, we revealed that the fate of ER glycoproteins is determined by a tug-of-war between folding and degradation factors in the ER. Our work has shed much light on a long-standing problem in the protein quality control system in the ER.

(P-100) Systematic Investigation of the Trafficking of Glycoproteins on the Cell Surface

Xing Xu[1], Kejun Yin[1], Ronghu Wu[*,1]

[1]*School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332, USA,*

Glycoproteins located on the cell surface play a pivotal role in nearly every extracellular activity. N-glycosylation is one of the most common and important protein modifications in eukaryotic cells, and it often regulates protein folding and trafficking. Glycosylation of cell-surface proteins undergoes meticulous regulation by various enzymes in the endoplasmic reticulum (ER) and the Golgi, ensuring their proper folding and trafficking to the cell surface. However, the impacts of protein N-glycosylation, N-glycan maturity, and protein folding status on the trafficking of cell-surface glycoproteins remain to be explored. In this work, we comprehensively and site-specifically studied the trafficking of cell-surface glycoproteins in human cells. Integrating metabolic labeling, bioorthogonal chemistry, and multiplexed proteomics, we investigated 706 N-glycosylation sites on 396 cell-surface glycoproteins in monocytes, either by inhibiting protein N-glycosylation, disturbing N-glycan maturation, or perturbing protein folding in the ER. The current results reveal their distinct impacts on the trafficking of surface glycoproteins. The inhibition of protein N-glycosylation dramatically suppresses the trafficking of many cell-surface glycoproteins. The N-glycan immaturity has more substantial effects on proteins with high N-glycosylation site densities, while the perturbation of protein folding in the ER exerts a more pronounced impact on surface glycoproteins with larger sizes. Furthermore, for N-glycosylated proteins, their trafficking to the cell surface is related to the secondary structures and adjacent amino acid residues of glycosylation sites. Systematic analysis of surface glycoprotein trafficking advances our understanding of the mechanisms underlying protein secretion and surface presentation.

(P-101) Proteasome inhibition leads to O-GlcNAc-mediated regulation of translation

Jie L. Tian[1], Farzad Eslami[1], Peng Zhao[1], Lance Wells[*,1], Gerald W. Hart[*,1]

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

Following 40 years of broad study, and over 5,000 publications, it has been established that O-GlcNAcylation (OGN) functions as a major nutrient and stress sensor to regulate enzyme activities, protein localization, protein associations, and protein degradation. OGN cycling is controlled by a sole pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), and

over 8,000 protein targets in human cells have been identified thus far. Most recently, emerging data suggests that OGN regulates translation and mRNA selection during nutrient stress. However, the mechanisms by which these processes are regulated by OGN are mostly lacking. Elucidating the molecular mechanisms by which OGN regulates translation is of critical importance in pathogenic conditions including diseases of ageing.

Our initial studies found that OGT and OGA are tightly associated with ribosomes and with translationally active polysomes. Remarkably, when cultured cells (e.g., HEK293F, MEF) were treated with a proteasome inhibitor, we found that the association of both OGT and OGA to ribosomes strikingly increased, resulting in elevated OGN of ribosome-associated proteins and translationally active polysomes. Importantly, the dynamic changes of OGT and OGA binding to the polysome differ in response to proteasome inhibition.

Multiple techniques were utilized to isolate O-GlcNAcylated proteins from ribosome and polysome fractions. O-GlcNAcomic analysis of the pooled TMT-tagged samples, following proteasome inhibitor treatment (0, 2, 4, 6h), detected 74/80 core ribosome subunits modified by OGN. Newly identified translation elongation factors, E3 ligases, mRNA splicers, and proteasome proteins also displayed significant changes in OGN levels after treatments. The MS/MS data indicate that OGN is involved in translation regulation at various levels while displaying extensive crosstalk with other post-translational modifications, such as ubiquitination, to regulate enzyme/protein activity during the stress response. GalT (Y289L) labeling, WGA pull-down, and immunoprecipitation assays confirmed that the chosen target proteins are indeed O-GlcNAcylated. Proteasome and OGN inhibitions also impacted the abundance of many proteins present in the ribosome and polysome fractions. Remarkably, both inducible OGT KO in MEF cells and knockdown of OGT using shRNA in HEK293F cells showed that diminished ribosome OGN nearly abolished ubiquitin binding to ribosome-associated proteins. Polysome profiles showed that OGT KO increases the quantity of non-translational ribosomes independently of MG132 treatment, suggesting that OGN has a role in regulating translation. Our data on the O-GlcNAcome of the translation machinery firmly paves the way for further mechanistic studies.

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(P-102) Comparison of mannosidase activity of EDEM2-TXNDC11 complex and MAN1B1

Shuichiro Oshita[1], Moe Yamashiro[1], Noritaka Hashii[2], Chia-Yen Liew[3], Yen-Ting Lin[3], Chi-Kung Ni[3], Hirokazu Yagi[4], Satoshi Ninagawa[1,5]

[1]Dept. of Bioresour. Sci., Grad. Sch of Agr., Kobe Univ, [2]NIHS, [3]Carbohydrate Mass Spectrometry Lab., Inst. of At & Mol Sci, Acad Sin, [4]Dept. of Struct. Bio & Biomol. Eng., Grad. of. Phar., Nagoya City Univ, [5]Biosig. Res. Ctr., Kobe Univ,

About one third of all proteins are biosynthesized in the endoplasmic reticulum (ER). These undergo various post-translational modifications, such as the addition of disulphide bonds and N-glycosylation (G3M9, which is composed of three glucoses, nine mannoses, two N-acetylglucosamine). N-glycosylation is performed on about 80% of proteins biosynthesised in the ER, where glucosidase removes three glucoses from the G3M9 to M9. When proteins have acquired their correct conformation, they enters the secretory pathway; if not, mannose trimming of their N-glycan proceeds and, they are eventually degraded by the cytoplasmic proteasome. Structurally aberrant proteins, which are produced by mistake due to failure of structure formation, pose a threat to living organisms because they cannot fulfil their regular function as proteins and, moreover, interact inappropriately with other proteins with normal functionality, resulting in toxicity. Therefore, the degradation of structurally abnormal proteins is of high physiological significance. Glycoprotein degradation is carried out by sequential trimming of mannoses from M9 to M8, and from M8 to M7, M6 and M5.

Although MAN1B1 was considered in the past as the mannosidase of the ER, endogenous MAN1B1 was shown to localize to the Golgi apparatus rather than to the ER (Pan et al., 2011 MBoC). It was shown that mannose trimming of the endoplasmic reticulum is mainly carried out by the EDEM Family (Ninagawa et al., 2014 JCB). Furthermore, one member of the EDEM Family, EDEM2, which performs mannose trimming from M9 to M8, was found to form a disulphide bond with TXNDC11, which exhibits a clear mannosidase activity from M9 to M8 *in vitro* for the first time (George, Ninagawa et al. 2020 eLife).

I have conducted research to address the issues arising from the aforementioned. The first issue is: 'What is the function of MAN1B1?' As a specific strategy here, I am working on the generation of MAN1B1-KO cells in human cultured cell lines, with the aim of elucidating the true function of endogenous MAN1B1. The content here will present the latest findings on the results of whole-cell glycan analysis and the impact on individual substrates, in comparison with the function of the EDEM family.

The second issue is that 'the mechanism of EDEM2 complex formation has been a major question but has not yet been clarified'. The aim here is to identify molecules that form disulphide bonds for the formation of the EDEM2-S-S-TXNDC11 complex. As a specific strategy, I proposed the PDI family of redox enzymes known to form disulphide bonds in the endoplasmic reticulum as candidate molecules. I am then trying to identify the molecules required for the formation of disulphide bonds linking EDEM2 and TXNDC11 by generating 10 gene disruption (KO) cell types.

The identification of the molecule is being approached by generating 10 types of gene-disrupted (KO) cells. I will present the new findings from these studies.

(P-103) Variations of the POGLUT2 and POGLUT3 putative consensus sequence produce aberrant O-glucose elongation on fibrillin-1

Nicholas R. Kegley[1], Atsuko Ito[1], Christina C. McArdle[1], Daniel B. Williamson[1], Robert S. Haltiwanger[1]

[1]*University of Georgia,*

Fibrillin-1 (FBN1) is an essential component of the Extracellular Matrix. FBN1 monomers associate into microfibril bundles, which form the basis of elastic tissue found in the heart and lung, and non-elastic tissue in the eyes and skeleton. Many missense mutations in the FBN1 gene are associated with Marfan syndrome (MFS), a common developmental disorder associated with symptoms including aortic aneurysm, ectopia lentis, emphysema, and arachnodactyly. FBN1 contains 47 Epidermal Growth Factor-Like (EGF) repeats, small domains characterized by the presence of 6 conserved cysteines and 3 disulfide bonds. Most of the EGF repeats are stabilized by bound calcium ions. Previously, FBN1 was discovered to be *O*-glucosylated by POGLUT2 and POGLUT3, two partially redundant glycosyltransferases that add an *O*-glucose modification to a serine within the putative consensus sequence of C^3-x-N-T-x-G-**S**-F/Y-x-C^4 found between cysteines 3 and 4 of an EGF repeat. The consensus amino acids are common between modified EGFs, but it is unclear if any of the residues are truly required for efficient *O*-glucose modification. Utilizing HEK293T cells, we overexpressed variants of an N-terminal FBN1 construct bearing EGF repeats 1–26 to determine if variation of the consensus residues affected *O*-glucosylation levels through a semi-quantitative mass spectral approach. Initially we performed an alanine scan of EGF15; the WT sequence is 100% modified by *O*-glucose monosaccharide. To our surprise, none of the putative consensus residues (except for S) were required for the addition of *O*-glucose, leading us to revise the consensus as C^3-x-x-x-x-x-S-x-x-C^4. While some variants displayed reduced relative *O*-glucose abundance, variants in the N, C^3 and C^4 positions were found to be elongated. The elongating glycosyltransferases included GXYLT1,2 and XXYLT1, which typically elongate POGLUT1 *O*-glucose with xylose. Elongation with galactose by B4GALT1 and subsequent sialylation was also observed. Following the EGF15 Ala scan, we made variants replicating MFS variants across other EGFs taken from the ClinVar and UMD databases. Generally, elongation was observed when residues that facilitate calcium coordination between EGFs (N, F/Y) were affected. Variation of residues that facilitate calcium coordination outside of C^3 and C^4 also produced elongation, suggesting a fold stabilized by calcium is protective against elongation not normally observed on WT FBN1. Elimination of disulfide bonds by replacing either C^3 or C^4 similarly resulted in elongation, suggesting that maintaining a WT fold is key for efficient monosaccharide modification. With the newly opened consensus, the possible number of POGLUT2 and 3 substrates has nearly doubled. The presence of elongation on MFS variants warrants further investigation into the effects of extra sugars on FBN1 function, which could play a role in the molecular mechanism of the disease. Supported by NIH grant HL161094.

(P-104) Subcellular localization analysis of Dystroglycan-specific modifying enzymes

Shinya Aso[1], Hirokazu Yagi[2], Kazutoshi Mori[3], Satashi Ninagawa[1,4]

[1]*Graduate School of Agricultural Sciences, Kobe University,* [2]*Graduate School of Pharmaceutical Sciences, Nagoya City University,* [3]*Graduate School of Sciences, Kyoto University,* [4]*Biosignal Research Center, Kobe University,*

Dystroglycan (DG) is a highly O-glycosylated protein and binds to extracellular substrates via its O-glycans. DG-specific modifying enzymes POMGNT2, B3GalNT2, and POMK work in the ER, while FKTN, FKRP, TMEM5, B4GAT1, and LARGE in the Golgi apparatus. These enzymes perform modification of DG in this order. These are all causative genes of a group of diseases collectively referred to as a-dystroglycanopathies. However, there have been no comprehensive studies on the major localization of these enzymes. Here, we expressed fluorescent fusion proteins of DG-specific modifying enzymes and observed their localization by confocal microscopy. POMGNT2 and B3GalNT2 localized to the ER as expected, but unexpectedly, POMK mainly localized in the Golgi apparatus. FKTN, FKRP and TMEM5 partially co-localized with GM130 (cis-/medial-Golgi marker) or TGN46 (trans Golgi marker), but their co-localization patterns were different. Localization of LARGE was overlapped with GM130, but not TGN46, indicating that major localization of LARGE is cis-/medial-Golgi. Though B4GAT1 did not co-localize with GM130 or TGN46, it co-localized with another trans-Golgi marker, GCC1. EndoH, which cleaves relatively simple N-linked glycans with oligo-mannose before passing through the Golgi for further modification, was used in the susceptibility experiments. All enzymes except B4GAT1 were sensitive to EndoH. B4GAT1 in cell lysate showed partial resistance to EndoH, although B4GAT1 co-immunoprecipitated with DG was EndoH-sensitive. These results indicated that all DG-specific modifying enzymes except B4GAT1 mainly localized to early secretory pathway. Our result revealed that POMK and B4GAT1 work at different localization from major one and support that modification of DG by its enzymes is completed at the cis-Golgi.

(P-105) Sex-Specific Differences in Healthy and Gestational Diabetes Mellitus Placental Proteins

Sarai Luna[1], Candice Tacconi[2], Stephanie Olivier-Van Stichelen[1]

[1]*The Medical College of Wisconsin,* [2]*University of Lille, France,*

Approximately 3–8% of all pregnant people in the United States are diagnosed with Gestational diabetes mellitus (GDM) each year. GDM is traditionally thought to emerge from placental endocrine dysregulations, but recent evidence suggests that fetal sex can also impact GDM development. Our research has uncovered a potential key to understanding and treating GDM. Indeed, our lab previously demonstrated significant differences in placental *O*-GlcNAc enzyme levels in GDM compared to healthy placentas (PMID: 34418053). *O*-GlcNAcylation is an atypical glycosylation that involves the addition of a single GlcNAc molecule to serine or threonine sites on proteins through an *O*-linked β-glycosidic bond. The addition is catalyzed by *O*-GlcNAc transferase (OGT) and reversed by *O*-GlcNAcase (OGA) through a hydrolytic cleavage.

Interestingly, we observed a decrease in *OGT* expression, specifically in male GDM placentas but not females. This correlated with clinical observations that those carrying a male fetus increase the risk of developing GDM by 39% and quickly transition to T2DM (type 2 diabetes mellitus) post-partum. Thus, understanding the molecular mechanisms through which sex modulates placenta physiology can help identify novel molecular targets for future clinical care, potentially revolutionizing the way we approach GDM treatment.

Based on the differences in *OGT* expression, we hypothesize proteins are differentially *O*-GlcNAc-modified in male vs. female and healthy vs. GDM placentas.

A total of 40 placenta samples, from healthy and GDM pregnancies (1:1) and from male and female fetuses (1:1) were obtained from the Medical College of Wisconsin Maternal Research and Placenta and Cord Blood Bank (MCW MRPCB) under IRB approval. For each placenta, we first characterized the global change in *O*-GlcNAcylation and demonstrated a significant decrease in the global *O*-GlcNAcylation profile in GDM male placentas. We then performed mapping of *O*-GlcNAcylated peptides that are differentially abundant in male vs. female and healthy vs. GDM placentas. Finally, we extracted ten significantly deregulated peptides in GDM *vs.* healthy placentas, according to sex, and confirmed by western blotting. In summary, we have produced a list of signature, sex-specific, and GDM-specific *O*-GlcNAcylated proteins to use as starting points for future studies investigating the role of *O*-GlcNAcylation in GDM.

While many research studies have mapped proteins in common cell lines and tissues, this study represents a significant advancement in using human tissues for *O*-GlcNAcomic studies in a pathological setting.

(P-106) Glycoproteomics and transport assay of ER-tethered protein Delta homolog 1 highlights the role of EGF domain-specific O-glycans in the secretory pathway

Yuko Tashima[1,2], Yohei Tsukamoto[1], Natsumi Tsukamoto[1], Yuji Kondo[1,2], Ehsan Uddin[1], Wakako Furukawa[1], Shiori Go[2], Hiroyuki Kaji[2], Hideyuki Takeuchi[1,2,3], Tetsuya Okajima[1,2]

[1]*Department of Molecular Biochemistry, Nagoya University Graduate School of Medicine, 65 Tsurumai-Cho, Showa-Ku, Nagoya, Aichi, 466-8550, Japan,* [2]*Institute for Glyco-core Research (iGCORE), Nagoya University, Furo-Cho, Chikusa-Ku, Nagoya, Aichi, 464-8601, Japan,* [3]*Department of Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka, 422-8526, Japan,*

In the endoplasmic reticulum (ER), O-glycosylation by O-fucose, O-glucose, and O-GlcNAc occurs in the epidermal growth factor-like (EGF) domains of secreted or transmembrane glycoproteins. Previous studies focusing on Notch receptors have revealed the pivotal role of these O-glycans in the cell surface expression of Notch or the secretion of truncated Notch fragments. Although it has been demonstrated that O-glycans stabilize individual EGF domains, their requirement in the secretory pathway remained unexplored. In this study, we used a delta-like 1 homolog (DLK1) containing six consecutive EGF domains as a model glycoprotein to investigate the role of EGF domain-specific O-glycans in the secretory pathway. Semi-quantitative site-specific glycoproteomics of recombinantly expressed DLK1 revealed multiple O-fucose and O-glucose modifications in addition to an unusual EOGT-dependent O-hexose modification. In parallel with the results of the secretion assay, inactivation of the glycosyltransferases modifying O-fucose and O-glucose but not the newly identified O-hexose perturbed the transport of DLK1 from the ER in the retention using the selective hooks (RUSH) system. Importantly, the absence of O-fucose did not result in an apparent loss of O-glucose modification within the same EGF domain and vice versa. Given that POFUT1 and POGLUT1 activities are dependent on the folded state of the EGF domains, these results suggest that DLK1 is properly folded before being transported to the cell surface. These findings, combined with proteomics data for interacting proteins of ER-retained DLK1, highlight the distinct roles of EGF domain-specific O-glycans in facilitating the transport of DLK1 from the ER to the cell surface.

(P-107) Forskolin reverses the O-GlcNAcylation dependent decrease in $GABA_AR$ current amplitude at hippocampal synapses possibly through action at the neurosteroid site on $GABA_ARs$

Shekinah Phillips[1,2], John C. Chatham[2], Lori McMahon[1]

[1]*Medical University of South Carolina,* [2]*Department of Neuroscience Charleston, SC, University of Alabama at Birmingham, Cell, Department of Developmental, and Integrative Biology and Pathology, Birmingham, AL,*

Recent work from our lab and others have shown that increasing protein O-GlcNAcylation depresses GABAergic transmission in hippocampus and dentate gyrus. Serine phosphorylation and O-GlcNAcylation are fundamental modulators of $GABA_ARs$, yet no study has examined whether these modifications interact to control $GABA_AR$ function. For decades, forskolin has been used to activate adenylyl cyclase to drive protein kinase A (PKA) dependent phosphorylation of AMPARs at excitatory synapses in hippocampus, leading to potentiation. Therefore, using forskolin, we sought to determine whether PKA dependent serine phosphorylation before and after pharmacologically increasing O-GlcNAcylation inhibits or amplifies the effect on $GABA_AR$ function. Using whole-cell recordings of evoked IPSCs (eIPSCS) from CA1 pyramidal cells and dentate granule cells in acute slices from 3–5 week old male and female rats, we bath applied forskolin (50 μM), either before (*CA1*:N = 9, n = 11; *Dentate:*N = 6, n = 6) or after (*CA1:*N = 10, n = 11; *Dentate:*N = 9, n = 10) bath application of glucosamine (5mM) and the O-GlcNAcase inhibitor, thiamet-G (1mM) to increase O-GlcNAcylation. In CA1 pyramidal cells, but not in dentate granule cells, forskolin induces a small but significant depression of baseline eIPSC amplitude, (10.4%), and had no effect on the magnitude of the O-GlcNAc dependent depression of eIPSC amplitude compared to control. However, in both CA1 and dentate, a prior increase in O-GlcNAcylation elicits a forskolin-dependent increase in IPSC amplitude (CA1:19.7%, Dentate:13.1%), thereby reversing the O-GlcNAc-induced synaptic depression. To confirm forskolin was working via PKA dependent phosphorylation, we used the PKA inhibitor, KT 5720 (N = 6, n = 8) and the adenylyl cyclase inhibitor SQ22536 (N = 7, n = 8), separately. Surprisingly, neither inhibitor prevented the forskolin dependent increase in $GABA_AR$ current amplitude following a prior increase in O-GlcNAcylation, indicating that this potentiation occurs through another mechanism. Interestingly, the inactive forskolin analog, 1,9-dideoxy Forskolin (N = 6, n = 8), used as a negative control also elicited a significant potentiation of eIPSC amplitude, also consistent with a non-PKA dependent mechanism. A previous study in carp amacrine-like cells (*Li& Yang, 2001)* found that forskolin can act directly at the neurosteroid site on $GABA_ARs$. Currently, we are testing the hypothesis that following a prior increase in O-GlcNAcylation, 5α-pregnane-3α,21-diol-20-one (THDOC;100 nM) application will mimic forskolin and increase the eIPSC, indicating that O-GlcNAcylation enhances access to the neurosteroid site on $GABA_ARs$.

(P-108) Extracellular ^{13}C-Fucose incorporation at a single glycosylation site of multiple proteins depends on N-glycan branching pattern. Golgi Apparatus has an unimagined complexity for substrate selection

Mayank Saraswat[1], Bobby G. Ng[1], Hudson H. Freeze[1]

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

Maturation and processing of N-glycans occurs in various compartments of the Golgi apparatus where donor nucleotide sugars, acceptor proteins and transferases transiently meet. Using ^{13}C-labeled fucose we previously showed that cells can distinguish whether GDP-Fucose originated from de novo synthesis, exogenous supplement, or was salvaged from internally degraded glycoproteins. Cells selectively recombine varying proportions of those separate sources based on the subcellular location, the specific protein, and the relevant fucosyltransferase. This suggests that GDP-fucose exists in at least 3 separate pools, as opposed to a single homogenous pool. We do not understand the sorting mechanisms.

To extend these observations further, we identified four individual proteins that are labeled to variable extents by fucose^{Ex} vs fucose^{endo}. We performed a time-series multiplexed N-glycoproteomic analysis of secreted glycoproteins labeled by ^{13}C-fucose^{Ex} in HepG2 cells. We show the effect of N-glycan branching on preferential utilization of fucose^{Ex} in a site-specific manner. We further establish the identity of glycan and proteins at defined glycosylation sites, showing differential utilization of fucose from different pools. These results suggest that the Golgi is far more complex than previously imagined, in some way selecting from different cytoplasmic GDP-Fucose pools. Our findings, using fucose, extends our understanding of monosaccharide metabolism, but likely can apply to other monosaccharides. Moreover, it reveals a previously unimagined complexity of substrate delivery and utilization for N-glycosylation in the Golgi.

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(P-109) OGT TPR Domain Variants Causal for OGT-CDG Have Altered Protein-Protein Interactions

N.L. Hitefield[1], H.M. Stephen[1], P. Zhao[1], L. Wells[1]

[1]*From the Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, 30605, United States of America,*

X-Linked Intellectual Disability (XLID) is a group of neurodevelopmental disorders attributed to mutations in the X-chromosome that affects 1 in 500 males in the United States. Our lab, and others, have reported missense mutations in the X-chromosome *OGT* gene found causal for an XLID disorder termed OGT Congenital Disorder of Glycosylation (OGT-CDG). OGT-CDG patients consistently present with intellectual disability (ID) and developmental delay alongside more variable features like facial dysmorphisms and clinodactyly. *OGT* encodes the essential human glycosyltransferase O-GlcNAc transferase (OGT), the sole enzyme responsible for the O-GlcNAc modification on serine and threonine residues of > 8,000 human nucleocytoplasmic proteins. OGT has roles in many cellular processes such as transcriptional regulation and neural development and function. The enzyme is composed of a N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal catalytic domain. The catalytic domain is responsible for the O-GlcNAc transferase activity of OGT, and the TPR domain provides OGT with enzyme targeting, substrate specificity, and protein-protein interactions. Previously, our lab characterized the first 5 variants identified in the TPR domain and found no uniform, appreciable defect in catalytic activity or thermal stability versus wild-type (WT). We therefore hypothesized the interactome of these OGT TPR variants was affected. Initial investigations of the WT OGT TPR domain interactome were done in HeLa cells via a BioID proximity labeling approach. The WT OGT TPR domain was fused with the promiscuous biotin ligase BirA∗ to label proteins nearby the TPR domain with biotin. Following neutravidin enrichment and shotgun proteomics, we identified 115 WT TPR domain interactors in HeLa cells including enrichment for proteins involved in transcriptional regulation and ID-related disorders. Our studies have since transitioned to disease-relevant neuroblastoma cells, including the female SH-SY5Y and male BE(2)-M17 lines, to compare interactors of the variant TPR domains compared to those of WT. TPR domain-only and full-length OGT constructs for WT and variant TPR OGT enzymes are fused to TurboID, a modified BirA∗ that reduces labeling time. These comparisons have identified multiple mechanistic targets for OGT-CDG dysregulated in the variant interactomes. Interactors are involved in transcriptional regulation and neural development and show an enrichment for proteins linked to ID-related disorders. We are in the process of validating WT OGT interactors that fail to interact with OGT-CDG variants as well as investigating the impact of loss of binding OGT on glycosylation and function of WT interactors.

(P-110) Chemotherapy induces the loss of sialic acid that promotes metastatic seeding of circulating tumor cells in breast cancer

Nurmaa Khund Dashzeveg[1], Yuzhi Jia[1], Youbin Zhang[2], Lorenzo Gerratana[2], Tsogbadrakh Dandar[1], Parastoo Azadi[3], William J. Gradishar[2,4], Massimo Cristofanilli[2,4], William A. Muller[4,6], Brian A. Cobb[5], Huiping Liu[1,2,4,6,]

[1]*Department of Pharmacology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA,* [2]*Department of Medicine, the Division of Hematology and Oncology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA,* [3]*Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, USA,* [4]*Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL, USA,* [5]*Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, USA,* [6]*Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA,*

Chemotherapy has been standard of care for triple negative breast cancer for decades; however, how chemotherapy evasion enables metastasis remains elusive and is one of the biggest challenges impacting patient survival in cancer clinics. Circulating tumor cells (CTC) are pivotal component of longitudinal liquid biopsies. Most of the CTCs are detected as a single cell, whereas small proportion of CTCs are found in multicellular clusters which possess 20–100 times higher metastatic potential than single CTCs and corelates with unfavorable survival in breast cancer patients. Here we observed that chemotherapy evasion is linked to the aggregation of CTCs in breast cancer. Chemo-evasive linked aggregated CTCs show specific loss of ST6GAL1-catalyzed α2,6-sialylation. Deficiency of ST6GAL1 promotes CTC cluster formation for metastatic seeding and enables cellular quiescence to evade paclitaxel treatment in metastatic breast cancer. Glycoproteomic analysis identified protein substrates of ST6GAL1, such as adhesion and stemness markers PODXL, ICAM1, ECE1, ALCAM1, CD97, and CD44, contributing to CTC clustering (aggregation) and metastatic seeding. As proof-of-concept, neutralizing antibody against one of the most significant contributors, PODXL, inhibits CTC cluster formation and lung metastasis exacerbated by paclitaxel treatment for breast cancer. This study sheds light in the evaluation of chemotherapy response and developed potential therapy approach against chemotherapy resistance.

(P-111) B4galt1 regulates the WNT-β-catenin axis to control Hematopoietic Stem and Progenitor Cells (HSPCs) fitness

Alejandro Roisman[1,2], Leonardo Rivadeneyra[1,2], Lindsey Conroy[3], Simon Glabere[1,2], Natalia Weich[1,2], George Steinhardt[1,2], Shikan Zheng[1,2], Anthony Veltry[1,2], Joseph Lau[4], Hartmut Weiler[1], Ramon Sun[2], Karin Hoffmeister[1,2,5]

[1]Versiti Blood Research Institute, Milwaukee, WI, [2]Translational Glycomics Center, Milwaukee, WI, [3]Department of Biochemistry & Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, [4]Roswell Park Comprehensive Cancer Center, Department of Molecular and Cellular Biology, Buffalo, NY, [5]Departments of Biochemistry and Medicine, Medical College of Wisconsin, Milwaukee, WI,

To achieve optimal blood cell numbers, intrinsic cellular programs and external guidance mechanisms are crucial. Glycans are pivotal in regulating cell maintenance, differentiation, and function. We recently discovered that the glycosyltransferase β-1,4-galactosyltransferase 1 (B4galt1) leads to dysplastic megakaryocytes (MK), impaired thrombopoiesis, and increased hematopoietic stem cells (HSC). We analyzed glycan composition in control and B4galt1 null ($B4^{-/-}$) femurs using MALDI-MSI to investigate their relationship with HSC function. In controls, we observed a distinct gradient of complex N-glycans, with higher expression at the distal ends and decreasing complexity towards the shaft. However, this gradient was absent in $B4^{-/-}$ femurs, where we detected increase immature N-glycans and a concurrent decrease in complex N-glycan structures ($P < 0.05$). Additionally, we observed aberrant O-glycosylation in $B4^{-/-}$ HSCs. This glycan composition in $B4^{-/-}$ mirrors cancer-associated glycan patterns. To further understand B4galt1's role in steady-state hematopoiesis, we examined the bone marrow (BM) immunophenotypic composition of $B4^{-/-}$ mice. Flow cytometry analysis revealed an expansion of immunophenotypically defined long-term (LT)-HSCs and multipotent progenitors (MPPs) in $B4^{-/-}$ BM. Additionally, $B4^{-/-}$ LT-HSCs showed higher expression of the platelet marker CD41, suggesting the presence of an expanded MK biased HSCs population. To investigate if the transcriptional landscape in $B4^{-/-}$ cells could explain the observed expansion, we conducted droplet-based single-cell RNA-seq. Transfer learning classification allowed us to identify an expansion of the transcriptional output related to LT-HSC and MPP2 signatures in $B4^{-/-}$ cells ($P < 0.05$). Further transcriptional analysis revealed an increased number of $B4^{-/-}$ cells expressing MKs specific markers (CD41, Pf4, GP9, Vwf, GP1ba), providing additional support for the expansion of $B4^{-/-}$ MK-biased LT-HSCs. Gene Set Enrichment Analysis (GSEA) of $B4^{-/-}$ LT-HSCs revealed enrichment in metabolism-associated pathways and cell cycle regulation. Critical differentiation processes at the stem cell level, such as cellular adhesion and lineage commitment, were downregulated (FDR < 0.05). Next, we classified LT-HSCs based on their cell cycle status and observed an increase in S phase in $B4^{-/-}$ cells. GSEA analysis unveiled upregulation of Wnt and Myc pathways in $B4^{-/-}$ LT-HSC CD41+ cells, further validated by immunofluorescence and immunoblot analysis. We then treated $B4^{-/-}$ cells with the Wnt inhibitor XAV939 which normalized cell numbers and caused a reduction in stem/progenitor compartments. These findings suggest that B4galt1 regulates HSC expansion, likely through the Wnt/β-catenin signaling pathway. Our data provide compelling evidence that B4galt1 regulates steady-state hematopoiesis, through its influence on glycosylation in the BM niche, highlighting the critical role of the B4galt1-Wnt-β-catenin axis in regulating HSC fitness.

(P-112) Characterization of a novel variant, R172H, of O-GlcNAc transferase associated with X-linked intellectual disability

Laura Holden[1], Johnathan Mayfield[1], Naomi Hitefield[1], Lance Wells[1]

[1]From the Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, 30605, United States of America,

X-linked intellectual disability (XLID) affects approximately one in 500 males in the USA. This group of neurodevelopmental disorders is caused by mutations in genes on the X-chromosome. O-GlcNAc Transferase (OGT) is one such gene that causes XLID in patients, which has been termed OGT-Congenital Disorder of Glycosylation (OGT-CDG). This neurodevelopmental disorder presents in patients with intellectual disability (ID) and developmental delay as well as variable other features including facial dysmorphisms and clinodactyly. OGT is responsible for the addition of O-GlcNAc onto serine or threonine residues of thousands of human nuclear and cytosolic proteins and is also responsible for the cleavage of the transcription factor HCFC1 in mammals. OGT and the O-GlcNAc modification play a role in numerous processes including transcription regulation, cell signaling, and normal development in mammals and other animal species. OGT has two domains: the Tetratricopeptide repeat (TPR) domain and the catalytic domain. The TPR domain is responsible for substrate selection and protein-protein interactions while the catalytic domain is responsible for the addition of O-GlcNAc onto target proteins and the cleavage of HCFC1. To date, variants of OGT responsible for OGT-CDG have been found to span the entire enzyme. Characterization of these variants by our lab and others has shown that catalytic domain variants are altered in their catalytic activity, and our lab has data to demonstrate that TPR domain variants have altered protein-protein interactions. With this in mind, we are characterizing a novel TPR domain

variant R172H in a male hemizygous patient who presents with mild ID and social communication disorder. *In silico* analyses suggest this variant is likely pathogenic. The arginine residue is highly conserved across model species including *C. elegans* and *D. melanogaster*, and a mutation to histidine would likely disrupt the interface between TPRs 4 and 5. Overexpression of this variant in mammalian cells shows no disruption of global O-GlcNAc levels or enzyme levels compared to wildtype OGT. Furthermore, we have shown that this variant does not have altered dimerization under physiological conditions using full-length mammalian-expressed recombinant enzymes and size exclusion chromatography. Our work going forward will fully characterize this variant in regards to enzymatic activity towards protein and peptide substrates as well as stability of the variant enzyme. We are currently pursuing efforts to characterize any altered protein-protein interactions via enrichment and mass spectrometry approaches. Furthermore, our lab is undergoing efforts to identify altered gene expression for this variant and others in efforts to identify a potential biomarker for OGT-CDG variants. As many more variants are identified and characterized including R172H, we will begin to understand the mechanisms behind this disorder as well as potential diagnostic markers and therapeutics for OGT-CDG patients.

Session 4: GAGs and Proteoglycans: Regulation and Function

(Key4-001) Decoding Glycosaminoglycan-Protein Interactions

Linda C. Hsieh-Wilson[1]

[1]*California Institute of Technology,*

Glycosaminoglycans (GAGs) interact with numerous proteins to regulate essential biological processes ranging from cell development and immune responses to viral invasion and neuroplasticity. We have developed a chemical platform for generating large heparan sulfate (HS) oligosaccharide libraries displaying comprehensive arrays of sulfation patterns. I will discuss how these diverse structures are providing new insights into the elusive 'sulfation code' of GAGs and the specificity of GAG-protein interactions. These comprehensive libraries enable the identification of unique sulfation sequences capable of modulating the activities of important growth factors and chemokines, as well as reveal intriguing parallels to DNA recognition.

(Key4-002) Loss of Layilin augments platelet activation via RAC1 in inflammatory bowel disease

Rebecca Mellema[1,2], Kimberly A. Queisser[1,2], Nansy Albtoush[1,2], Lydia Smith[1,2], 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,*

Over 3 million Americans suffer from inflammatory bowel disease (IBD) which increases the risk of thrombosis up to 16-fold during active disease as compared to the general population. Thrombosis is fatal in almost one third of IBD patients, and is one of the leading causes of death in IBD. Platelet activation closely correlates with disease activity and severity in IBD, suggesting that platelets play an important, but overlooked role in disease pathogenesis. Platelets are specialized cells essential for hemostasis that also function as crucial effectors of inflammatory and immune responses, balancing the signals which activate these responses from those that preserve quiescence. Our recent work suggests that platelets play an early, protective role in colitis by regulating immune cell adhesion by remodeling the glycosaminoglycan hyaluronan (HA), but this protective mechanism is lost in IBD and models of murine colitis. HA metabolism requires platelet activation and exposure of hyaluronidase-2 at the platelet surface, but the mechanism by which platelets recognize HA during is not known.

Using a combination of biochemical assays, animal models, and patient cells, we have identified the C-type lectin layilin as a receptor for HA expressed by platelets that also functions as a novel regulator of platelet activation. Our data suggests that layilin interactions with HA function to restrain platelet activation in response to protease-activated receptor (PAR) agonists in human platelets (as measured by active integrin αIIbβ3 and P-selectin), and that binding of layilin with either HA or monoclonal antibody is a weak, reversible inhibitor of platelet aggregation. Loss of layilin in a C57BL/6 background leads to significantly increased sensitivity to g-coupled protein receptor activation (PAR4-agonist, thrombin, and thromboxane A2) as compared with healthy controls. Surprisingly, layilin deficient platelets demonstrate reduced aggregation in response to low dose thrombin or collagen when compared with controls, suggesting a potential defect in clot formation. We show that layilin-deficient platelets are primed for activation by basal activation of a RAC1/PAK1/merlin signaling axis, and accordingly, direct targeting of RAC1 with chemical inhibitors show that layilin KO platelets are resistant to inhibition upon thrombin stimulation. Finally, using

platelets isolated from IBD patients or mice subject to experimental colitis, we demonstrate that layilin levels are reduced in patient platelets at the mRNA and protein levels, and correspond with increased basal RAC1-GTP and platelet activation responses. Together, these data suggest that layilin-HA interactions act as an endogenous inhibitor of platelet activation and that in settings of chronic inflammation, this mechanism is lost and can directly contribute to increased platelet activation and thromboinflammation associated with IBD.

(Key4-003) Glycosaminoglycan biosynthesis as a therapeutic vulnerability of breast cancer minimal residual disease

Amulya Sreekumar[1,2], Michelle Lu[1,2], Biswa Choudhury[4], Tien-chi Pan[1,2], Dhruv K. Pant[1,2], Matthew R. Lawrence-Paul[1,2], Christopher J. Sterner[1,2], George K. Belka[1,2], Takashi Toriumi[1,2], Brian A. Benz[1,2], Matias Escobar-Aguirre[1,2], Francesco E. Marino[1,2], Jeffrey D. Esko[4], Lewis A. Chodosh[1,2,3]

[1]Department of Cancer Biology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA, [2]Abramson Family Cancer Research Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA, [3]Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA, [4]Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093, USA,

Breast cancer is the leading cause of cancer-related deaths in women worldwide. Mortality results primarily from recurrent metastatic disease that can occur several years or decades after successful treatment of the primary disease. Recurrences are seeded by residual tumor cells (RTCs), which constitute minimal residual disease following therapy. RTCs are thought to persist in a therapy-refractory, dormant state. Therefore, determining the mechanisms that enable dormant RTC survival following therapy and their eventual resumption of growth is critical for preventing disease-related mortality.

To elucidate the mechanisms enabling RTC dormancy, we utilized an inducible MMTV-*Her2/Neu* genetically engineered mouse model that recapitulates breast cancer progression in patients. We isolated cells from primary tumors, residual lesions, and recurrences to derive RTC-specific gene expression signatures, which identified multiple extracellular matrix-related modules selectively enriched in RTCs. To determine which of these components functionally impacts RTC survival and subsequent breast cancer progression, we performed an extracellular matrix modules-focused CRISPR-Cas9 loss-of-function screen in vivo.

We found that B3GALT6, an enzyme essential for proteoglycan assembly, promotes RTC fitness. B3GALT6 is required for the synthesis of a tetrasaccharide linker that connects the core protein to heparan sulfate and chondroitin sulfate glycosaminoglycans to generate functional proteoglycans. Consequently, the knockout of B3GALT6 specifically impairs glycosaminoglycan synthesis, extension, and modification. We validated the findings from the CRISPR-Cas9 screen and confirmed that B3GALT6, and by extension, glycosaminoglycan synthesis, promotes dormant RTC survival and is required for subsequent recurrence.

To identify the subset of proteoglycans that confer a fitness advantage to RTCs, we performed mass spectrometry and observed upregulated synthesis and overall sulfation of heparan sulfate proteoglycans, but not chondroitin sulfate proteoglycans, in these cells. We determined that B3GALT6-mediated biosynthesis of the heparan sulfate glycosaminoglycans predicts poor patient outcomes, promotes tumor recurrence by enhancing dormant RTC survival in multiple contexts, and does so via a B3GALT6-heparan sulfate/HS6ST1-heparan 6-*O*-sulfation/FGF1-FGFR2 signaling axis. These findings identify a role for B3GALT6 in cancer and suggest that glycosaminoglycan biosynthesis is a selective vulnerability of dormant RTCs.

(Key4-004) The role of heparan sulfate modification on neurexin1 in synapse development

Peng Zhang[1], Qin Xu[1], Jing Ma[1], Donovan Whitfield[1], Destini Weller[1]

[1]Department of Neurosciences, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106,

Proper synaptic function relies on a dedicated assembly of pre- and postsynaptic proteins. Neurexin1 is a principal presynaptic organizing protein essential for synaptic function. Recently, we unexpectedly discovered that a complex glycan, heparan sulfate, attached to the extracellular domain of neurexin1. Blocking the attachment of heparan sulfate glycan to neurexin1 results in severe deficits in synaptic ultrastructure and function in mice. Furthermore, we identified a growth factor, pleiotrophin, as a novel ligand that binds directly to neurexin through its heparan sulfate glycan. Our results suggest a new molecular model that neurexins' heparan sulfate glycan servers a "sugar" platform to recruit growth factors, such as pleiotrophin, to synapses, which promote the development and function of synapses.

(PT-025) *UGDH* Sequence Variants Highlight the Importance of its Allosteric Regulation by Nucleotide Sugars During Vertebrate Development

Po-Nien Lu[1], Nolan Ross-Kemppinen[2], Richard Steet[1], Zachary A. Wood[2], Heather Flanagan-Steet[1]

[1]JC Self Research Institute, Greenwood Genetic Center, Greenwood SC 29646, [2]Department of Biochemistry and Molecular Biology, University of Georgia, Athens GA 30603,

Precisely regulating the balance of nucleotide sugar precursors is critical to control output of downstream pathways involved in glycan biosynthesis. One class of glycans, the glycosaminoglycans (GAGs), are comprised of repeating disaccharide units containing one uronic sugar and one amino sugar, typically glucuronic acid (GlcA) and either N-acetyl galactosamine (GalNAc) or N-acetyl glucosamine (GlcNAc). UDP-glucuronic acid (UDP-GlcA), one of the vital nucleotide sugars, is produced by UDP-glucose 6-dehydrogenase (UGDH). Mutations which dysregulate UDP-GlcA production are associated with abnormal development of multiple systems, including cardiac, cartilage, and neuronal tissues. All metazoan UGDHs utilize an allosteric feedback mechanism where another nucleotide sugar involved in GAG synthesis, UDP-xylose, inhibits activity. Previous work has shown that an intrinsically disordered tail (ID-tail) that increases the affinity for UDP-xylose by 7-fold is exclusively conserved in vertebrate UGDHs. New studies suggest that UGDH amino acid 127 also contributes to this regulatory mechanism. Phylogenetic analyses suggest amino acid 127 co-evolved with the ID-tail, with vertebrates bearing a threonine rather than the valine found in invertebrates. Kinetic analyses of human UGDH show that the Thr127Val substitution reduces the affinity for UDP-xylose by 3-fold. Removing the ID-tail in the Thr127Val background further reduces UGDH sensitivity to UDP-Xylose inhibition 21-fold. To investigate how variant forms of human UGDH (bearing a Thr127Val with or without the tail) impact vertebrate development, we compared the ability of wild type and variant containing enzymes to rescue cartilage and cardiac phenotypes in *ugdh*-deficient zebrafish. Consistent with previous zebrafish studies, wild type enzyme restored normal cartilage morphology in 58% of *ugdh*-deficient larvae, while enzyme bearing the Thr127Val substitution only rescued cartilage defects in 33% of *ugdh*-deficient larvae. Surprisingly, enzyme with the Thr127Val substitution that also lacks the ID tail rescued cartilage defects in 73% of the *ugdh*-deficient larvae scored, suggesting it may be a better (albeit less regulated) enzyme. We hypothesize that the double variant enzyme, which is less sensitive to UDP-xylose inhibition, produces more UDP-GlcA than the wild type enzyme - resulting in enhanced rescue of *ugdh* deficiency. Analytical studies are underway to investigate this and whether altering UDP-GlcA levels impacts other nucleotide sugars (like UDP-GlcNAc). Analyses of *UGDH* variants that alter enzyme efficiency and allosteric regulation provides a unique opportunity to understand how modulating the sugar nucleotide pools impacts downstream glycosylation pathways and tissue development.

(PT-027) Elucidating the interaction mechanisms between the proteins involved in chondroitin sulfate biosynthesis

Daniel Tehrani[1], Digantkumar Chapla[1], Chin Huang[1], Zhifeng Zheng[1], Kelley W. Moremen[1]

[1]Biochemistry and Molecular Biology Department, University of Georgia, Athens GA 30603,

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) attached to a core proteoglycan (PG) that is found in the extracellular matrix of connective tissues like cartilage, bone, skin, and ligaments. There it serves several varied roles including membrane flexibility, strength, and cell signaling. CS, and a related PG, heparan sulfate (HS), are repeat disaccharide polymers that are both initiated through the formation of a tetrasaccharide linkage region attached to a variety of PG core proteins, like bikunin, syndecan, and glypican. The committed step in CS polymer synthesis results from the addition of a single β1,4-N-acetylgalactosamine (GalNAc) to the linker tetrasaccharide by the enzymes CSGALNACT1 or CSGALNACT2, instead of being initiated down the HS pathway by the addition of an α1,4-N-acetylglucosamine (GlcNAc) by EXTL3. Following this committed step, the CS polymer is elongated by alternating addition of β1,4GalNAc and β1,3-glucuronic acid (GlcA) residues by four proteins, CHPF1, CHPF2, CHSY1, and CHSY3, to yield CS chains as long as 100 monosaccharide units. However, their direct role in CS elongation is unknown. Sequence alignments and structural insight using AlphaFold Multimer reveal that all CHPFs and CHSYs contain both a Glycosyltransferase Family 7 (GT7)-like GlcA transferase domain and a GT31-like GalNAc transferase domain separated by a cystatin-like domain. Here, we create and analyze various heterodimer complex models to predict which oligomeric complexes might exist in the CS pathway. We then express these complexes to show that a combination of one CHPF and one CHSY can form viable complexes with one another that are capable of elongating CS chains, while individual proteins are not stably expressed. Furthermore, we generated domain-specific catalytically inactive mutants to elucidate co-polymerase activity of these proposed bifunctional, multidomain proteins. We also propose that heterocomplex formation is essential to provide stability and mechanistic features required for CS elongation. Further kinetic analysis on these enzyme complexes will provide an understanding of the roles of these proteins in CS biosynthesis and an improved understanding of the protein-specific interactions involved in CS elongation.

(PT-029) Unmasking the role of heparan sulfate proteoglycans in α-synuclein pathology

Saumya Digraskar[1], Jaewon Huh[1], Piyali Das[1], Abigail Songer[1], Kevin Graciano[1], Rita Cowell[1], Jeffrey Esko[2], Laura Volpicelli-Daley[1], Patricia Aguilar Calvo[1]

[1]*Department of Neurology, The University of Alabama at Birmingham, Birmingham AL,* [2]*Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA,*

Synucleinopathies, including Parkinson's disease (PD) and dementia with Lewy bodies (DLB), are characterized by the aggregation and neuronal accumulation of the α-synuclein protein (α-syn) leading to debilitating motor, cognitive and psychiatric dysfunction. While the trans-synaptic spread of α-syn aggregates between neurons is well-established, extracellular α-syn has a significant role in disease progression. However, the receptors responsible for α-syn cellular uptake are not yet fully understood. Heparan sulfate proteoglycans (HSPGs) are glycoproteins that colocalize with α-syn deposits in PD brains and may act as receptors for the internalization of α-syn. We hypothesize that HSPGs modulate α-syn pathology by mediating the *neuronal* internalization and aggregation of α-syn. Since HSPGs interact with ligands through the negatively charged sulfate groups, we investigated how decreasing HS sulfation impacts α-syn pathology and disease progression in mice injected with α-syn aggregates. First, we incubated neurons and astrocytes from mice conditional knockout for the *N-Deacetylase/N-Sulfotransferase-1* (*Ndst1*$^{f/f}$), the enzyme responsible for the N-sulfation of HS chains, with α-syn aggregates to determine the role of HSPGs sulfation in α-syn cellular incorporation. We found that reducing HS sulfation almost completely prevented the *neuronal* incorporation of α-syn aggregates, while reduced *astrocytic* incorporation by 50%. We next investigated whether HSPGs sulfation modulate the *in vivo* propagation of α-syn pathology. We injected α-syn aggregates into the striatum of mice that express lower *neuronal* HS sulfation and their wild-type (WT) littermates and assessed their behavioral abilities with the progression of α-syn pathology at 7 weeks post-injection. We found that mice expressing lower *neuronal* HS sulfation had reduced pathological α-syn accumulation than WT littermates. Moreover, HS mutants showed lower levels of anxiety than their WT counterparts, which suggests that decreasing HS sulfation slows down α-syn pathology. Interestingly, reducing neuronal HS sulfation has been shown to accelerate prion clearance and extend lifespan by 50% in mice. Hence, to investigate the impact of reduced *neuronal* HS sulfation on α-syn clearance *in vivo*, we stereotaxically injected Zirconium89-radiolabeled α-syn aggregates into the striatum of mice with reduced or physiological HS sulfation and tracked the spread of the radiolabeled α-syn using PET scanning. We found that mice with reduced HS sulfation had less radioactive signal in the brain than WT mice after 22 hours, suggesting that reducing HS sulfation enhances the clearance of α-syn. Altogether, our results show how sulfated HS modulates α-syn uptake, spread and clearance, impacting the progression of α-syn pathology. Based on these findings, our research highlights the impairment of the HSPG:α-syn interactions as a potential therapeutic strategy for synucleinopathies.

(PT-031) A Genome-Wide CRISPR Screen Uncovers a Novel Role for Mucin-Type O-Glycosylation in Heparan Sulfate Assembly

Xiaolin Dong[1,2], Kaleigh E. Gosnell[2], 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,*

Heparan sulfate (HS) is a long, linear cellular polysaccharide that plays a crucial role in numerous biological processes, including cell signaling, development, and tissue homeostasis. Dysregulation of HS biosynthesis is implicated in a variety of pathophysiological conditions, such as the rare bone disorder Multiple Hereditary Exostoses (MHE). HS polysaccharides are essential for normal skeletal development and chondrocyte function at the growth plate, and disruptions in HS biosynthesis due to Exostoxin-1 (EXT1) or Exostoxin-2 (EXT2) mutations contribute to the formation of exostoses in MHE. Despite the importance of HS in development and human disease, the regulatory mechanisms controlling HS biosynthesis remains largely unknown. To investigate potential regulatory mechanisms of HS assembly, we utilized genome-wide CRISPR screening assays to explore the upstream and downstream regulators of EXt1, a Golgi-localized enzyme essential for HS assembly. We established an endogenous EXT1 reporter cell line by integrating a green fluorescent protein (GFP) at the C-terminus of endogenous *EXT1* in human chondrocytes via CRISPR/Cas9 gene editing. We subsequently conducted genome wide CRISPR screening assays to identify regulatory factors that impact *EXT1* expression. The top hits from the EXT1 screens included β-1,3-galactosyltransferase 1 (C1GALT1) and its molecular chaperone, Cosmc (C1GALT1C1), both of which are involved in Core 1 mucin-type *O*-glycosylation. Our data reveals that loss of COSMC or C1GALT1 alters *EXT* expression, HS composition, and fibroblast growth factor-mediated cell signaling pathways, highlighting the intricate crosstalk between HS biosynthesis and mucin-type *O*-glycosylation. Interestingly, knockout of COSMC or C1GALT1 in chondrocytes also selectively altered HS proteoglycan expression and presentation at the cell surface. The interplay between HS biosynthesis and mucin-type O-glycosylation may reveal new mechanisms regulating HS levels. Elucidating specific regulatory pathways controlled by O-glycosylation that impact HS biosynthesis may provide critical insights for developing targeted therapies for MHE and other congenital disorders of glycosylation.

(P-113) Characterization of heparin interactions with *Clostridioides difficile* toxins

Fuming Zhang[1], Shaohui Wang[2], Jiyuan Yang[1], Keith Fraser[3], James M. Gibson[3], Chunyu Wang[3], Jonathan S. Dordick[1], Anastasia Tomatsidou[2], Robert J. Linhardt[1], Lianchun Wang[4], Xingmin Sun[2,*]

[1]*Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA,* [2]*Department of Molecular Medicine, Morsani College of Medicine, University of South Florida Tampa, FL 33620, USA,* [3]*Department of Biological Science, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA,* [4]*Department of Molecular Pharmacology and Physiology, Morsani College of Medicine, University of South Florida, Tampa, FL 33620, USA,*

Clostridioides difficile (*C. difficile*) infection (CDI) is a life-threatening healthcare-associated infection occurring worldwide. *C. difficile* toxins (toxin A and toxin B) are the major virulence factors, causing CDI-related diarrhea and complications. Recent studies have shown that sulfated glycosaminoglcans (GAGs) are involved in mediating the cellular entry of these toxins. While GAG-toxin interactions have been previously reported, their binding kinetics and the structure features of glycans that facilitate toxin interaction have not been thoroughly investigated. In this study, we performed direct binding measurement using surface plasmon resonance (SPR) to characterize the kinetics of interactions between heparin and the toxins. Both toxin A and toxin B bind to heparin with high affinity (K_D = 3.3 nM and 13.5 nM, respectively). SPR competition assay showed that both toxin A and B prefer binding to longer heparin chains and that all sulfation on the heparin chain is important for the heparin-toxin interaction. Finally, an *in vitro* assay showed that heparin and non-anticoagulant heparin inhibit the cell rounding caused by toxin A in HeLa cells.

(P-114) Utilizing Isotopic Labeling and HILIC-MS for Glycosaminoglycan Structural Determination and Sulfation Pattern

Stephanie Archer-Hartmann[1], Amrita Basu[1], Mehrnoush Taherzadeh Ghahfarrokhi[1], Ryan J. Weiss[1], Parastoo Azadi[1]

[1]*Complex Carbohydrate Research Center, UGA, Athens, GA,*

Glycosaminoglycans (GAGs) are crucial biomolecules that play roles in various physiological and pathological processes. Changes in their sulfation patterns determine how GAGs interact with different proteins, affecting signaling pathways, specificity of molecular recognition, and biomechanical properties. Alterations in GAG sulfation patterns have been associated with various diseases and disorders. Thus, developing precise and readily accessible analytical techniques is crucial for studying GAGs across various fields. Here we present a method combining isotopic reducing end labeling with hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC LC/MS) for the quantitative analysis of heparan sulfate (HS) and chondroitin/dermatan sulfate (CS/DS) glycosaminoglycans. Lyase-generated disaccharides and commercial standards were tagged on the reducing end with aniline stable isotopes (^{12}C6 and ^{13}C6), thus enabling absolute quantification of each HS and CS disaccharide in complex biological samples and without prior cleanup. In addition, we show that this method can be adapted for the detection and quantification of non-reducing ends of glycosaminoglycans, providing a scheme for the assessment of disease-specific biomarkers in biological samples. Overall, this method demonstrates enhanced sensitivity and specificity compared to previous isotopic labeling or fluorophoric workflows and avoids the use of ion pairing agents, which can be problematic in some analytical contexts. By integrating HILIC separation with isotopic labeling, our approach presents a robust and user-friendly tool for GAG analysis, advancing the understanding of GAG biology.

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(P-115) Selective 2-Desulfation Induced by Mild Acid Hydrolysis in Tetrasaccharide-Repeating Sulfated Fucans During Oligosaccharide Production

Marwa Farrag[1,2,†], Seon Beom Kim[1,†,‡], Sushil K. Mishra[1,§], Sandeep Misra[1], Joshua S. Sharp[1,3,4], Robert J. Doerksen[1,4], Vitor H. Pomin[1,4]

[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 Chemistry and Biochemistry, University of Mississippi, Oxford, Mississippi, USA,* [4]*Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, Mississippi, USA,*

[†]Both authors have made equal contributions

[‡]Current affiliation: College of Pharmacy, Chungbuk National University, Cheongju, Republic of Korea

[§]Current affiliation: Department of Biomedical Engineering, University of Mississippi Oxford, Mississippi, USA

Sulfated fucans (SFs) from echinoderms, such as sea cucumbers and sea urchins, present linear and regular sulfation patterns within defined oligosaccharide building blocks. The high molecular weights of these polymers pose a problem in advanced structure-activity relationship studies for which derived oligosaccharides are more appropriate tools for investigation. However, enzymes capable of specifically depolymerizing SFs, fucanases, are not very common. Scarce abundance and unknown catalytic activities are additional barriers to exploiting fucanases. Oligosaccharide production by controlled chemical reactions such as mild acid hydrolysis then becomes a convenient strategy. As a consequence, physicochemical studies are necessary to understand the structural modifications caused on SFs by this chemical hydrolysis. Hence, in this work, we subjected three tetrasaccharide-repeating SFs from sea cucumbers, *Isostichopus badionotus* (IbSF), *Holothuria floridana* (HfSF), and *Lytechinus variegatus* (LvSF) to mild acid hydrolysis for oligosaccharide production. Interestingly, a selective 2-desulfation reaction was observed in all three SFs. Through our study, we indicate that selective 2-desulfation is a common and expected phenomenon in oligosaccharide production by mild acid hydrolysis of SFs, including those composed of tetrasaccharide-repeating units.

(P-116) Human hippocampal aging links biphasic changes in 6S chondroitin sulfate isomer expression with shifts in cognition and whole brain grey matter volume

Aarun Hendrickson[1], David Tovar[2], Andrew Bender[3], Griffin Rangel[1], Jaden Le[1], Cristiana Meuret[1], Asmit Kumar[1,4], Kendra Francis[4,5], Miklos Guttman[1], C. Dirk Keene[4], Caitlin Latimer[4], Michael W. Schwartz[4], Jarrad M. Scarlett[4,5], Kimberly M. Alonge[1,4]

[1]*Department of Medicinal Chemistry, University of Washington, Seattle, WA,* [2]*Department of Psychology, Vanderbilt University, Nashville, TN,* [3]*Department of Cognitive Science, University of California San Diego, San Deigo, CA,* [4]*Department of Medicine, University of Washington, Seattle, WA,* [5]*Department of Pediatric Gastroenterology and Hepatology, Seattle Children's Hospital, Seattle, WA,*

Brain aging associates with shifts in extracellular matrix (ECM) chondroitin sulfate-glycosaminoglycans (CS-GAGs), including pericellular CS-GAG matrix enmeshments of cognitive and memory-forming neurocircuits in the hippocampus. The biological function of these CS-GAG matrices are dictated by sulfate modifications to their repeating glucosamine and N-acetylgalactosamine disaccharide units, including the mono-sulfated 6S-CS isomer. Although previous work in mice shows that high 6S-CS expression promotes neurocircuit plasticity prior to the critical period in neurodevelopment, this isomer is dramatically reduced in the adult mouse brain. However, the translatability of these findings is currently unknown, so we first asked whether age-associated changes in hippocampal 6S-CS isomer expression in mice translates to humans. Here, we show that mice (7d–2.2 y, 119 samples, 51% M) and humans (0d–95 y, 57 samples, 49% M) showcase the highest abundance of 6S-CS after birth, including 7d-old mice (53.6%) and humans < 1 month of age (20.3%). We then observed a steady decline in mouse hippocampal 6S-CS expression at ages 14 d (28.4%), 21 d (21.4%), 28 d (17.7%), 42 d (11.5%), 3 m (7.3%), 6 m (5.6%), 1 y (5.5%), 1.5 y (3.3%), and 2.2 y (3.5%), which mirrors previously reported changes in whole brain isolates. Surprisingly, although the human hippocampus exhibits an initial decline from < 1 month of age (20.3%) to 4–5 y (11.7%), 6–10 y (9.5%), 11–20 y (8.6%), and 21–30 y (6.6%), we found that human hippocampal 6S-CS abundance began to rise again from 31–50 y (8.2%), 51–80 y (8.5%), and 80–95 y (8.9%), (ages 21–95 y; $R^2 = 0.21$, $p = 0.0135$). Since increased 6S-CS expression in the adult brain associates with matrix de-stability and neurocircuit dysfunction, we next determined whether the steady increase in hippocampal 6S-CS expression starting in midlife correlates to previous reports showing decreases in both cognition (fluid intelligence) and brain structure (grey matter volume (GMV)) starting at 20–30yr of age. Using representational similarity analysis (RSA) coupled with Bayesian optimization, we found a significant correlation between 6S-CS expression and changes in GMV throughout the lifespan of humans (Spearman rho = 0.57, $P < 0.01$). Moreover, changes in 6S-CS expression also significantly correlated with the loss of cognition starting in midlife ($R^2 = 0.15$, $p = 0.0149$). Overall, we now predict that age-related changes in CS-GAG composition in humans may drive macrostructural shifts in brain volume and age-associated loss in cognition.

(P-117) Hyperglycemia Induced Hyaluronan Matrix in Diabetic Atherosclerotic Cardiovascular Diseases

Andrew J. Wang[1], Aimin Wang[2], Vincent C. Hascall[2]

[1]*Department of Medicine, New York University Grossman School of Medicine, New York, NY, USA,* [2]*Department of Biomedical Engineering, Cleveland Clinic, Cleveland, OH, USA,*

Diabetes is a metabolic disease often associated with hyperglycemia, resulting in extensive and chronic inflammation, which is the most prominent factor leading to atherosclerosis and subsequent cardiovascular disease [1]. The hyperglycemia-induced hyaluronan matrix has been suggested to play an essential role in the development and progression of diabetic atherosclerotic

cardiovascular disease by mediating the proinflammatory responses of infiltrating monocytes and macrophages in the arterial vasculature [2,3]. The underlying mechanism(s) is not fully understood. Here, our studies show that there are: 1) activation of smooth muscle cells and 2) formation of hyaluronan matrix in the media of rat aortic vessel walls at 8 weeks after the onset of diabetes. At this time, thickening of the glomerular basement membrane, one of the earliest lesions of diabetic nephropathy, becomes prominent. We also tested the response of aortic smooth muscle cell (SMC) cultures for monocyte adhesion in response to hyperglycemia. When the near-confluent, growth-arrested SMCs were stimulated by serum to re-enter into cell growth cycles, hyperglycemic glucose (25.6 mM) significantly increased hyaluronan in the cell matrix with a concurrent robust increase in adhesion of U937 monocytes to SMC cultures. The monocytes primarily bind directly to hyaluronan-based structures in vitro. These responses, independent of increased extracellular osmolality, required growth stimulation of SMCs by serum and activation of protein kinase C. Interestingly, these glucose-induced cellular responses were not observed in the prior passage of SMCs in the presence of heparin. Further, when incubated with the viral mimetic, poly I:C under normal glucose condition, these heparin-pretreated SMCs still increased cell-associated hyaluronan and exhibited hyaluronan-mediated adhesion of monocytes. These results suggest that high glucose and viral mimetic induce both production of extracellular hyaluronan structures and the monocyte adhesion via distinctly different intracellular signaling mechanisms. These results support the hypothesis that hyperglycemia in the arterial vasculature activates and diverts SMCs into a metabolically stressed pathway that induces the synthesis of a hyaluronan matrix. This recruits inflammatory cells and establishes a chronic inflammatory process that mediates the development and progression of atherosclerosis leading to cardiovascular disease in diabetes.

References

Verges B. *Atherosclerosis*. 2024:**394**:117158.
Hascall VC. *J Biol Chem*. 2019:**294**:1690–1696.
Wang A et al. *Proteoglycan Res*. 2023:**1**:e7.

(P-118) An Alzheimer's Disease-associated heparan sulfate glycan inhibits synapse development and function

Qin Xu[1], Leanne Auyeung[1], Zhangjie Wang[2], Jian Liu[2], Peng Zhang[1]

[1]*Department of Neurosciences, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, USA,* [2]*Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA,*

Synaptic loss is one leading early deficit in Alzheimer's disease (AD) and is most closely correlated with cognitive decline in AD patients. Yet the mechanisms underlying synapse elimination remain poorly understood. Our recent findings reveal a sevenfold increase in a specific 3-O-sulfated heparan sulfate (HS) in the brains of AD patients, though its role in synapse development and function is unknown. In this study, we demonstrate that a synthetic tetradecasaccharide (12-mer-19), which contains the same 3-O-sulfated domain found in AD patients, significantly disrupts synapse development and function. In contrast, a non-sulfated 12-mer does not affect synapse development. Our findings uncover the deleterious impact of AD-associated HS sulfated glycans on synapse development and function, highlighting that the sulfated groups rather than the HS backbone are critical. These results suggest that extracellular complex glycans, alongside numerous protein factors, could play an important yet underappreciated role in regulating synaptic loss and dysfunction in the early stages of AD.

(P-119) Determining the interaction site of Cocaprin 1 from *Coprinopsis cinerea* to *Listera innocua* peptidoglycan

Sandeep K. Misra[1], Nika Janež[2], Jerica Sabotič[2], Joshua S. Sharp[1]

[1]*University of Mississippi, University, MS,* [2]*Jozef Stefan Institute, Jamova cesta 39, Slovenia,*

Lectins are proteins that reversibly bind to carbohydrates and glycoconjugates, often with relatively low affinity. Understanding how they recognize carbohydrates selectively is important for fully understanding the importance of lectin-carbohydrate interactions, which play many important roles in a wide range of biological processes. Protein Cocaprin 1 (CCP1) purified from *Coprinopsis cinerea* showed sequence similarity to known lectins suggesting that it may be a lectin. To verify that CCP1 is a lectin and to identify the peptidoglycan binding site of this protein, as well as any conformational changes to this protein upon binding, we employed hydroxyl radical protein footprinting.

The samples, containing 5 μM of the protein, 1 mM adenine in 50 μM phosphate buffer, pH 7.8, with or without 15 μM peptidoglycan, and 100 mM hydrogen peroxide, were irradiated using a Fox Protein Footprinting System (GenNext Technologies). The samples were collected into a quench solution containing 35 mM methionine amide and 0.3 mg/mL catalase. After quenching, the samples were heat denatured, divided into two equal parts, and digested with trypsin and chymotrypsin separately for 16 hr. 0.1% formic acid was added to the samples and the samples were run on an Orbitrap Exploris mass spectrometer coupled with a Dionex Ultimate 3000 nanoLC system (Thermo Fisher, CA).

The dosimetry of the samples, as measured by adenine absorbance, with or without peptidoglycan was comparable, indicating no need for compensation for radical scavenging. Both trypsin and chymotrypsin were used, achieving more than 83% sequence coverage of the protein. We detected a total of nine peptides oxidized and one peptide was detected with no oxidation by the hydroxyl radicals. Three peptides (corresponding to protein residues 39–48, 49–58, and 82–104) were significantly protected from oxidation modification ($p \leq 0.05$) in the presence of peptidoglycan, with the other peptides showing no significant changes in oxidation upon peptidoglycan binding. The regions that are protected form a spatially compact area that we propose as the peptidoglycan binding site. No regions of exposure upon peptidoglycan binding were detected. Current docking simulations and mutagenesis studies are underway to validate the importance of these residues in protein-peptidoglycan interactions.

(P-120) The Syndecan-1 Hug Mediates Hepatic Triglyceride-rich Lipoprotein Remnant Clearance in Mice

Chelsea Nora[1,*], Ferdous Anower-E-Khuda[2,*], Tiffany H.C. Lee[1,2], Vineet Tummula[1], Jeffrey D. Esko[2,4], Philip L.S.M. Gordts[1,4,]

[1]Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA, USA, [2]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, USA,, [3]Department of Ophthalmology, University of California, San Diego, La Jolla, CA, USA, [4]Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093, USA,

Syndecan-1 (SDC1) is a Type I transmembrane protein with three heparan sulfate (HS) chains near its N-terminus, playing a critical role in triglyceride (TG) and triglyceride-rich lipoprotein (TRL) metabolism. SDC1 knockout mice (Sdc1-/-) exhibit TG accumulation and impaired TRL remnant clearance, highlighting SDC1's unique function compared to other receptors like LDLR and LRP1. SDC1 binds TRLs via its HS chains, specifically clearing TRLs enriched with apoC-III, apoE, and apoA-V. Our study investigated the structural and functional aspects of SDC1 in TRL clearance using Adeno-Associated Virus (AAV) mediated liver reconstitution in Sdc1-/- mice. We found that only two of three HS chains are essential for TRL clearance, with chains at serine 45 and 47 being particularly critical. Constructs lacking these HS chains failed to rescue the clearance phenotype. Furthermore, the SDC1 ectodomain alone is insufficient for TRL clearance, and proper cleavage by proteases is necessary. The intracellular domain, containing an endocytic MKKK motif, is crucial for SDC1 function. Mutations preventing SDC1 dimerization or lacking the endocytic motif resulted in defective TRL clearance, despite normal cell surface expression. Chimeric SDC1 and SDC4 constructs confirmed the necessity of the SDC1 intracellular domain for TRL endocytosis. These findings elucidate the mechanisms behind SDC1-mediated TRL clearance and suggest that targeting SDC1 could offer new therapeutic strategies for managing plasma TG levels and reducing cardiovascular disease risk.

Session 5: Gaining Insights into Glycoenzymes and Glycoconjugates with: Genetic Tools

(Key5-001) Mucin engineering for health and disease understanding

Matthew Paszek[1]

[1]Cornell University,

Mucins are sugar and protein co-polymers that are well recognized for their roles in protecting cell and tissue surfaces. Recent advances in mucin engineering afford new opportunities to dissect the multifaceted functions of mucins in health and disease. Because the mucin polypeptide backbone is genetically encoded, sequence-specific mucins with defined physical and biochemical properties can be fabricated using custom gene synthesis. Furthermore, delineation of O-glycosylation pathways has provided a roadmap for tuning the glycan structures that decorate the mucin backbones. I will discuss how these strategies, in combination with super-resolution optical imaging, have contributed to new understanding of native mucins in the regulation of membrane dynamics, cellular signaling, and intercellular interactions. We have found that densely grafted mucins on the cell surface form a

polymer brush layer whose physical properties can be predicted using classical theories from polymer physics. This understanding has enabled us to program intercellular interactions through mucin engineering. Cancer cells similarly coopt these principles to evade immune surveillance through construction of a protective mucin layer. These results raise the exciting prospect that outcomes with immunotherapy may be improved when combined with strategies that disrupt the protective mucin layer of cancer cells. Overall, our results suggest new possibilities to control the intercellular interactions that define immune function and multicellular life more broadly.

(Key5-002) Surface display technologies for the high-throughput screening of glycan binding proteins and glycosyltransferases

Sriram Neelamegham[1]

[1]*State University of New York, Buffalo, NY,*

The presentation will describe a recently developed mammalian cell surface-display technology where either lectins or glycosyltransferases are presented in a context similar to how they are in the Golgi. Once displayed, mutations can be implemented on them to study the effect of site-specific modification. Screening 1600 different mutations, we will present the development of a lectin that specifically binds sialylated core-2 glycans. The binding profile of this new lectin to blood cells, normal human tissue and cancers will be described. Results from the same platform will also be presented for the screening of multiple sialyltransferases including previously uncharacterized Carbohydrate-active enzymes (CAZymes) from multiple species. This effort yields, novel biocatalysts with improved activity compared to natural homologs.

(Key5-003) Models and mechanisms of O-GlcNAc transferase intellectual disability

Daan M.F. van Aalten[1]

[1]*Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark,*

The O-GlcNAc post-translational modification of intracellular proteins is essential for embryogenesis, development and brain function. Our knowledge of how O-GlcNAcylation regulates protein function and associated pathways is limited. An exciting new 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 now been reported, it is not understood how these mutations are mechanistically linked to the neuro-developmental deficits seen in the patients.

I will describe common symptoms (intellectual disability, epilepsy, developmental delay) in a cohort of > 65 unpublished patients, and 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. Following on from a range of omics approaches, we have discovered the RNA helicase and intellectual disability protein DDX3X as a potential driver of this syndrome.

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 are 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.

(Key5-004) The Use of Glycoengineered Cell Lines and Tissue Models to Study Essential Functions of Specific Glycans in Viral Infections

Hans H. Wandall[1]

[1]*Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark,*

Breakthroughs in O-glycobiology have advanced alongside the development of new technologies, such as improvements in mass spectrometry and the genetic engineering of mammalian cell lines. We have previously demonstrated how genetic engineering, combined with a human organotypic platform, has enabled a systematic investigation of the roles of distinct glycan types in cellular differentiation and tissue formation. Furthermore, this model allows for the testing of new glycan-based treatments, including the selectivity of potent immunotherapies targeting cancer-associated truncated O-glycans, such as Tn. In this presentation, we will focus on the use of our glycoengineered keratinocyte library to uncover the roles of specific glycans at different stages of viral infections, using herpes simplex virus type 1 (HSV-1) as an example. We show the importance of cellular glycosaminoglycans and glycosphingolipids in HSV-1 attachment, N-glycans in entry and spread, and O-glycans in propagation. While altered virion surface structures have minimal effects on early interactions with wild-type cells, mutations at specific O-glycosylation sites affect glycoprotein surface expression and function. In conclusion, the data demonstrate the utility of genetic engineering in elucidating the roles of specific viral and cellular carbohydrate structures.

(PT-002) Activity-based genome-wide CRISPR-Cas9 pooled screening for modifiers of lysosomal glucocerebrosidase uncovers candidate risk factors for Parkinson disease

Pierre-André Gilormini[1,2], Vinod Udayar[3,4], Julien Bryois[3], Alexandra Gehrlein[3], Xi Chen[2], Stephanie Sonea[1], Sha Zhu[2], Matthew C. Deen[2], Nadia Anastasi[3], Alan E. Murphy[5,6], Nathan Skene[5,6], Manuela M.X. Tan[7], Jon-Anders Tunold[7], Filip Roudnicky[4], Wilma D.J. van de Berg[8], Lasse Pihlstrøm[7], David J. Vocadlo[1,2], Ravi Jagasia[3]

[1]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC V5A 1S6 Canada, [2]Department of Chemistry, Simon Fraser University, Burnaby, BC V5A 1S6 Canada, [3]Roche Pharma Research and Early Development, Neuroscience and Rare Disease discovery and translational area, Roche Innovation Center Basel, 4070 Basel, Switzerland, [4]Roche Pharma Research and Early Development, Therapeutic Modalities, Roche Innovation Center Basel, 4070 Basel, Switzerland, [5]UK Dementia Research Institute at Imperial College London, United Kingdom, [6]Department of Brain Sciences, Imperial College London, United Kingdom, [7]Department of Neurology, Oslo University Hospital, Oslo, Norway, [8]Department of Anatomy and Neurosciences, Section Clinical Neuroanatomy and Biobanking and Life Sciences, Vrije Universiteit Amsterdam, Amsterdam, 1081 HV, Netherlands,

Parkinson's disease (PD) is a complex, multifactorial, neurodegenerative disease. The most common genetic risk factors for PD are mutations in the gene *GBA1*, which encodes for the lysosomal enzyme glucocerebrosidase (GCase). This enzyme is responsible for the hydrolysis of the glycolipids glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph). Loss of function and consequent lysosomal accumulation of these lipids are thought to drive disease. Interestingly, *GBA1* mutations show variable penetrance, which strongly suggest the existence of genetic modifiers of GCase activity beyond those already known to aid in trafficking of this enzyme to lysosomes. To identify candidate genes that modify lysosomal GCase activity, we implemented a live cell GCase activity-based flow cytometry assay to enable genome-wide screening using CRISPR-Cas9.

We used our dark-to-light fluorescent-quenched substrate LysoFQ-GBA to measure the effect of genetic disruption of each gene on lysosomal GCase activity. This approach allowed us to robustly identify a set of genes which, when knocked-out, significantly decrease GCase lysosomal activity. Validation of a subset of these genes supports the precision and accuracy of our screening methods. Among approximately 50 candidate modifiers influencing GCase activity, we found significant enrichment of genes linked to development and progression of PD through genome-wide association studies (GWAS). Notably, we identified two lysosomal lipid transporter genes including those encoding the lysophospholipid transporter SPNS1 and the cholesterol transporter NPC1. Subsequent bioinformatic analysis uncovered an allele of *SPNS1* that is associated with increased risk of PD. Focusing our efforts on SPNS1 and NPC1, we showed that their genetic disruption does not affect GCase protein levels nor total activity from lysates but impairs its lysosomal function. Collectively, these data suggest that dysfunction of many PD associated genes converge to impact lysosomal GCase activity and thereby likely act upstream of this enzyme to contribute to disease pathogenesis. A better understanding of the impacts of *SPNS1* and *NPC1*, as well as the other GCase modulators identified here, should help unravel the important, yet complex, relationship between *GBA1* and PD.

(PT-004) O-mannosylation of E-cadherin in an invasive cancer spheroid model

K. Madunić[1], I. Signe Bohse Larsen[1], W. Tian[1], L. Povolo[1], A. Halim[*,1]

[1]Copenhagen Center for Glycomics, University of Copenhagen,

Cadherins and protocadherins constitute a family of adhesion proteins that enable cell-cell adhesion though their extracellular cadherin (EC) domains. EC-domains undergo extensive and abundant O-mannose glycosylation by TMTC1-4 enzymes. Loss of E-cadherin expression is a prominent feature of epithelial to mesenchymal transition (EMT), however, epithelial cancer cells

can also undergo EMT and form distant metastases without downregulation of E-cadherin expression. Inherited deficiencies in *TMTC1-4* genes severely affect embryonic development and have been identified as GWAS candidates for cancer development. However, the functional roles and impact of EC-domain O-Man glycosylations remains understudied in cancer models.

Using CRISPR-Cas9 genetic engineering, we knocked out (KO) *TMTC1-4* to examine the functional effects of O-mannosylation in an ovarian cancer cell line model, and specifically focused on O-Man dependent functions of the classical E-cadherin molecule. We confirmed global loss of O-mannosylation on cadherins and protocadherins by mass spectrometry before establishing WT and *TMTC1-4* KO spheroids. Interestingly, the absence of O-mannosylation on cadherins and protocadherins did not impact spheroid formation in culture. However, *TMTC1-4* KO led to increased expression of the mesenchymal marker vimentin and enhanced invasion when spheroids were embedded in a collagen basement membrane matrix. Notably, cell surface expression of E-cadherin was not influenced by *TMTC1-4* KO, indicating that the functional state of E-cadherin, and potentially other cadherins and protocadherins, are fine-tuned by O-Man glycosylation.

To further explore the connection between O-mannosylation and E-cadherin in cancer, we developed a panel of high-affinity monoclonal antibodies (MAbs) that recognize different E-cadherin EC-domains, which were validated in cells by KO of *CDH1* as well as *TMTC1-4*. Our results confirm that E-cadherin traffics to the cell surface independent of O-Man glycosylation, but also reveal loss of MAb binding to specific EC domains in *TMTC1-4* KO cells. Thus, it further indicates that E-cadherin adopts distinct conformational states that may influence E-cadherin functions and ultimately cell-cell interactions in an O-Man dependent manner. Taken together, these results indicate that O-mannosylation influences cadherin structure-function relationships, which we are currently exploring with CryoEM techniques and immunohistochemical staining of cancer patient tissues.

(PT-006) Revolutionizing Homogeneous Glycosylation: The SUGAR-TARGET Platform

Dilyara Sadykova[1], Elli Makrydaki[1], Cleo Kontoravdi[1]

[1]*Department of Chemical Engineering, Imperial College London, London, UK,*

The biotherapeutic industry has grown significantly, driven by glycoproteins like monoclonal antibodies, which are crucial in treating autoimmune diseases, cancers, viral infections, influenza, and bacterial and fungal infections. Glycoproteins are synthesized in the rough Endoplasmic Reticulum and processed in the Golgi apparatus through complex enzymatic glycosylation. This non-templated process, characterized by enzyme promiscuity, substrate competition, and variable glycosylation site occupancy, leads to product heterogeneity, which can provoke immune responses and impact therapeutic efficacy. Controlling glycosylation has been challenging, with existing methods being laborious, costly, and requiring extra purification steps. To address these issues, we introduce the novel cell-free SUGAR-TARGET platform, designed for sequential glycosylation reactions to achieve bespoke homogeneous glycoproteins [1]. The SUGAR-TARGET platform uniquely addresses enzyme promiscuity by spatial-temporal separation of glycosylation reactions, avoiding enzyme competition and undesirable heterogeneous structures, thus producing tailored homogeneous glycoproteins. Controlled glycosylation in the SUGAR-TARGET platform is enabled by immobilized enzymes, achieved through a one-step immobilization/purification process involving strong non-covalent interactions between our *in vivo* biotinylated enzymes and streptavidin-coated beads. We recreated a natural glycosylation cascade to humanize proteins from various systems, achieving near-homogeneous glycoforms. The platform is straightforward, modular, reusable, and can produce consistent glycan structures for functional and clinical evaluations. To make the SUGAR-TARGET platform industry-applicable, we optimized reaction conditions for glycosylation involving immobilized plant and human N-acetylglucosaminyltransferase I (GnTI) and β-1,4-galactosyltransferase (GalT), studying their kinetics. We reduced glycosylation reaction time with the GnTI enzyme from 16h to 1h and established that the immobilized GalT enzyme could be reused for up to three cycles without losing efficiency. Additionally, for the first time, we produced *in vivo* biotinylated *Drosophila melanogaster* Golgi α-1,2-Mannosidase II (ManII) in *Escherichia coli*, explored its application, and successfully incorporated it into the SUGAR-TARGET cascade. Looking forward, we aim to further optimize reaction times for the Man II enzyme, refine pH conditions for GnTI, ManII, and GalT enzymes to streamline reactions, and explore their reuse to reduce costs. The SUGAR-TARGET platform represents a significant advancement in glycoengineering, offering a scalable and efficient solution for producing homogeneous glycoproteins, ultimately enhancing the therapeutic potential of biopharmaceuticals.

References

Makrydaki E, Donini R, Krueger A, et al. Immobilized enzyme cascade for targeted glycosylation. *Nat Chem Biol*. 2024:**20**:732–741. doi: https://doi.org/10.1038/s41589-023-01539-4.

(PT-008) Disruption of individual protein glycosylation pathways in *Pseudomonas aeruginosa* results in distinct phenotypes for motility, biofilm formation, and antibiotic resistance

Stefan Schulze[1], Chenxin Wang[1], Lorelei Robinson[1], Aldo T. Trejos[2], Maya Quaranta[1], Elena Fuentes Solano[1], Diya Mathai[1], Samuel Kopelev[1], Katherine Palmatier[1], George Trekell[1], Sherry Kuchma[3], George O'Toole[3], Mechthild Pohlschroder[4]

[1]*Rochester Institute of Technology, Rochester, NY, USA,* [2]*University of Washington, Seattle, WA, USA,* [3]*Dartmouth College, Hanover, NH, USA,* [4]*University of Pennsylvania, Philadelphia, PA, USA,*

Pseudomonas aeruginosa is an opportunistic, bacterial pathogen that poses a significant threat to human health, especially in patients with cystic fibrosis or burn wounds. Its ability to form strong biofilms and the rapid spread of antibiotic resistance often complicate the treatment against this pathogen. While four separate *O*-glycosylation pathways have been described for *P. aeruginosa*, a systematic analysis of the roles of protein glycosylation in various biological processes is missing so far, and the glycoproteome of this organism remains to be elucidated. Here, we have generated knockout mutants of key enzymes in two glycosylation pathways in the same wild-type background and assessed their phenotypes through various cell biological assays. Interestingly, distinct phenotypes for each mutant were observed with regards to swimming, swarming, and twitching motility, biofilm formation, and antibiotic resistance. These results indicate, (i) that protein glycosylation is crucial for a range of biological process which are closely linked to the pathogenicity of *P. aeruginosa*, (ii) that multiple glycosylation pathways are active in the same wild-type strain, and (iii) that their roles in cellular functions are distinct from each other. In addition, we have performed mass spectrometry-based proteomics and identified a glycoproteome that is at least an order of magnitude larger than the handful of glycoproteins that have previously been described for *P. aeruginosa*. Together, these results highlight the complexity and biomedical importance of protein glycosylation in bacteria.

(P-121) Parallel CRISPR screens identify functional modules of glycosylation

C. Kimberly Tsui[1], Andrew Dillin[1]

[1]*Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA 94720, USA,*

Glycan synthesis follows highly interconnected paths within a cell, and modulation of a single step can drastically alter the cellular glycome. This modulation occurs through the activity and localization of biosynthetic enzymes, the trafficking of substrates, and the organization of cellular organelles, representing a complex functional network the cell uses to regulate the glycome for both homeostasis and in response to perturbations such as cellular stress. To address the identity and organization of this expanded network of glyco-regulation, we have developed a magnetic sorting-based screening approach to rapidly perform parallel genome-wide CRISPR knockdown screens for the cell surface presentation of glycan epitopes. We will use this to uncover genetic determinants for high mannose, core fucose, bisecting GlcNAc, highly branched N-glycans, α2,3-, and α2,6-sialic acids. By leveraging this large screening dataset, we can not only identify novel genes regulating the glycome but also extract functionally linked gene modules that will allow us to begin to understand how the cell controls what glycans are presented at a systems level.

We have demonstrated the efficacy of this approach in pilot experiments, where we found that clustering glycosylation phenotypes of dozens of gene knockdowns allowed us to obtain groups of functionally related genes and aided the characterization of novel glycosylation regulators. The larger scale analysis using the genome-wide screening data will uncover comprehensive genetic regulatory networks that can be used to infer relationships between the glycan biosynthetic enzymes, Golgi, and intracellular trafficking regulators, as well as predict the function of previously uncharacterized genes that have similar glycosylation phenotypes as known genes. This work will greatly contribute to our understanding of how glycosylation phenotypes are linked and uncover many genes previously not linked to glycosylation.

(P-122) Significance and function of Secretion of Fucosyltransferase FUT8

Seita Tomida[1], Rebeca Kawahara[2], Yasuhiko Kizuka[1,3]

[1]*The United Graduate School of Agricultural Science, Gifu University, Gifu, Japan,* [2]*Institute for Glyco-core Research (iGCORE), Nagoya University, Nagoya, Japan,* [3]*Institute for Glyco-core Research (iGCORE), Gifu University, Gifu, Japan,*

Alpha1,6-fucosyltransferase (FUT8) is the glycosyltransferase that transfers fucose to the reducing end GlcNAc of *N*-glycan to form core fucose. Core fucose is involved in various diseases, including COPD progression, melanoma metastasis, and severity of

COVID-19. While the physiological and pathological functions of core fucose are well studied, the regulatory mechanisms of its biosynthesis, FUT8 activity, and expression remain largely unknown. Several Golgi-localized glycosyltransferases including FUT8 are known to be cleaved by proteases before secretion to outside cells. In fact, FUT8 activity in blood has been detected, indicating that FUT8 is secreted. The significance of secreted glycosyltransferases includes the regulation of intracellular expression levels and extracellular glycosylation under specific conditions. However, the functions and secretion mechanisms of these extracellular glycosyltransferases remain unclear. Recently, Signal peptide peptidase-like 3 (SPPL3) which is the transmembrane protease belonging to the SPP/SPPL family has been identified as the major enzyme that cleaves multiple glycosyltransferases. However, any specific protease responsible for cleaving FUT8 has not yet been identified. Therefore, in this study we aimed to elucidate the regulatory mechanism and significance of FUT8 secretion.

First, to identify the enzyme which is responsible for the secretion of FUT8, knockout cells for each member of SPP/SPPL family were generated by the CRISPR-Cas9 system. The enzymatic activity and abundance of intracellular and secreted FUT8 from each knockout (KO) cell were measured using HPLC and Western blotting. As a result, the expression levels of intracellular FUT8 were not changed in both wild type (WT) and KO cells but the activity of extracellular FUT8 from SPP and SPPL3 KO cells was both decreased to roughly 40% of that of WT cells. In addition, the secretion of FUT8 was further decreased by double knocking out of SPP and SPPL3 compared to single KO cells. Second, to elucidate the cleavage mechanism of FUT8 in detail, the *N*-terminal amino acid sequence of secreted FUT8 was determined by Edman degradation. As a result, the *N*-terminus of secreted FUT8 is located in the linker sequence of the stem region. Furthermore, deletion of this linker region causes reduction of secretion. Finally, to elucidate the significance of secretion, expression levels of core fucose was investigated using FACS and glycoproteomic analysis. The FACS results showed that core fucose levels were slightly decreased in SPPL3 KO. These results indicate that both SPP and SPPL3 are required for the FUT8 secretion, and the linker region in the stem region is essential for cleavage by SPP and SPPL. In addition, we are trying to see how secretion of FUT8 is implicated in the core fucose levels on each *N*-glycosylated protein.

(P-123) Sequential Quality Control Pathways Reduce Mutant Cosmc Expression in *Cosmc*-CDG

Rajindra P. Aryal[1], Aditya Ramanujan[1], Camille Bucci[1], Michael Cselovszky[1], Maxence Noel[1], Florian Erger[2,3], Bodo Beck[2,3], Richard D. Cummings[1]

[1]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA, [2]Institute of Human Genetics, University Hospital Cologne, Faculty of Medicine, University of Cologne, Cologne, Germany, [3]Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany,

A heritable mutation in *COSMC (*Ala20Asp, A20D), which we found to occur in patients with a congenital disorder of glycosylation (*COSMC*-CDG) results in a remarkable loss of Cosmc protein expression, but how this occurs mechanistically is largely unclear. Cosmc is a molecular chaperone in the ER that regulates formation of a single client protein, the T-synthase, which is the sole enzyme in the Golgi apparatus responsible for all complex O-glycan production in mammalian glycoproteins. The A20D mutation occurs within the core hydrophobic signal sequence embedded in the transmembrane domain (TMD) of Cosmc; the mutation reduces the hydrophobicity of the signal sequence and is known to impact interactions with signal recognition particle (SRP), which helps target nascent polypeptides to the ER for continued translation. We found that a newly discovered Regulated Aberrant Protein production (RAPP) pathway, which monitors the signal sequence emerging from the ribosome exit tunnel and SRP interaction, is involved in degrading mutant A20D-Cosmc mRNA. Our results demonstrate the severity of engineered mutations aimed to lower the hydrophobicity within the core hydrophobic region of the internal signal sequence within the TMD of Cosmc and is specifically correlated with decreased Cosmc protein expression and T-synthase function. Additionally, the stability of the A20D Cosmc mRNA in patient cells depends on translation. We also demonstrated that mutant A20D-Cosmc, as monitored by its disulfide bond formation and N-glycosylation status, can functionally enter the ER. However, analysis of Cosmc complexes showed a significantly lower level of aberrant A20D-Cosmc assemblies compared to WT, indicating mutant Cosmc fails to correctly assemble and is degraded by the ER-associated degradation pathway or ERAD. Our results elucidate a novel pathway in which mutations within the core hydrophobic signal sequence of the TMD within a membrane protein can trigger multiple quality control pathways that regulate their biogenesis.

(P-124) Combined Systemic and Conditional *Asgr1* and *Asgr2* Deficiency for Investigating the Role of the Ashwell-Morell Receptor in Development and Physiology

Robert Fraumeni[1], John Hintze[1], Jamey D. Marth[1]

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

The Ashwell-Morell receptor (AMR) was the first isolated cell receptor protein and first identified mammalian lectin presently defined as the prototypical C-type lectin receptor. The AMR is expressed at high levels specifically among hepatocytes where it functions in the clearance from circulation of desialylated glycoproteins and blood components including platelets. The mature mammalian AMR is composed of two lectin receptor protein subunits encoded by the closely linked *Asgr1* and *Asgr2* genes. Mouse strains deficient in either *Asgr1* or *Asgr2* have been previously produced by others and have been studied for multiple decades. We have previously reported the role of the AMR in controlling the half-lives via endocytic clearance of circulating blood proteins and components as they lose sialic acid with increased molecular age. We have further observed similar glycoprotein ligand repertoires often with a dependence on both chains for endocytic clearance. The full scope of AMR function is however unknown as compensation for the loss of one chain by the other is further implicated. Moreover, the proposed roles and mechanisms of AMR function in other cell types, including the Kupffer cell, could not be addressed previously due to the close physical proximity of the *Asgr* genes on mouse chromosome 11 thereby preventing simple inter-breeding to produce offspring lacking both AMR chains. Herein we describe the development of a novel mouse model wherein *Asgr2* has been constitutively mutated to a null allele whereas *Asgr1* is engineered as a conditional-null allele with both modifications on the same chromosome haplotype. By employing this genetically engineered mouse model, including the use of Cre-loxP mutagenesis previously conceived and co-developed by this laboratory, we can render mouse strains either systemically deficient in both *Asgr1* and *Asgr2* from conception or conditionally deficient among select cell types, including in an inducible manner. In this way, we aim to discover both the broader functions as well as the more selective roles of the AMR to achieve mechanistic advances and expand our understanding of how the AMR controls physiology. Our current findings establishing and implementing this research plan will be presented.

(P-125) Fringe glycosyltransferase overexpression alters osteoclast differentiation

Jason W. Ashley[1]

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

Notch signaling is a direct contact pathway that is essential for proper differentiation of osteoclasts. Osteoclasts, responsible for the breakdown of bone matrix during skeletal remodeling, differentiate from macrophage-like precursors, which fuse together to form multinucleated giant cells that can be histochemically identified via staining or quantifying activity of the enzyme Tartrate Resistant Acid Phosphatase (TRAP). Although transgenic and chemical inhibition studies have highlighted the necessity of Notch signaling for osteoclast maturation, the specific roles of the four Notch receptors (Notch1, Notch2, Notch3, Notch4) and how they are modulated remain unclear. Signaling through Notch receptors is modified by the Fringe family of O-linked fucose-specific N-acetylglucosaminyltransferases, which alter receptor ligand preference and receptor signaling potency. The three members of the fringe family, Lunatic Fringe (LFNG), Manic Fringe (MFNG), and Radical Fringe (RFNG) demonstrate different patterns of sugar addition to the Notch receptor extracellular domain. We have found expression of all three enzymes in osteoclasts and their precursors, and we hypothesize that Fringe proteins fine-tune Notch signaling to facilitate proper osteoclast differentiation. As part of our on-going investigation of Notch signaling in osteoclast biology, we have overexpressed members of the Fringe family in primary mouse bone marrow-derived macrophages and evaluated their osteoclastogenic potential. Our current results suggest that overexpression of either LFNG or MFNG increases osteoclast precursor fusion compared to control, and the resultant osteoclasts were larger and demonstrated robust actin organization at their peripheries. This increased osteoclastogenesis was accompanied by increased TRAP activity in the cells' conditioned media. In contrast to the enhancement seen with LFNG and MFNG, RFNG-overexpressing cells showed no significant difference in TRAP activity and no apparent alteration in osteoclast size compared to controls. In addition, RFNG-overexpressing osteoclasts presented with less apparent rings of actin, though more quantitative microscopy is required to ascertain significance of this observation. These initial findings suggest both supportive and inhibitory roles for the Fringe-mediated glycosylation in osteoclastogenesis. Future work will investigate the mineral-dissolving activities of these cells, Fringe-differential patterns of Notch receptor glycosylation in osteoclasts and their precursors, and how Fringe activity impacts osteoclastic gene expression downstream of Notch signaling.

(P-126) Disruption of O-GlcNAc homeostasis is a common feature of O-GlcNAc transferase intellectual disability variants

Huijie Yuan[1,2], Conor W. Mitchell[1], Andrew T. Ferenbach[1], Maria Teresa Bonati[3], Agnese Feresin[4], Paul J. Benke[5], Queenie K.G. Tan[6], Daan M.F. van Aalten[1,2]

[1]*Department of Molecular Biology and Genetics, Aarhus University, Aarhus, DK,* [2]*Division of Molecular, Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee, UK,* [3]*Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy,* [4]*University of Trieste, Department of Medicine, Surgery and Health Sciences, Trieste, Italy,* [5]*Joe DiMaggio Children's Hospital, 1131 North 35th Avenue, 2nd Floor, Hollywood, FL 33021,* [6]*Department of Clinical Genomics, Mayo Clinic, Rochester, NY, USA,*

O-GlcNAcylation is an essential protein modification catalysed by O-GlcNAc transferase (OGT). Missense variants in *OGT* are associated with a novel intellectual disability syndrome called OGT Congenital Disorder of Glycosylation (OGT-CDG). The biological mechanisms by which OGT missense variants give rise to this heterogeneous syndrome are not understood, and there is no unified system for dissecting pathogenic from non-pathogenic variants. Here, we develop a double fluorescence strategy in mouse embryonic stem cells to measure disruption of O-GlcNAc homeostasis, giving an exploitable difference between wild type and catalytically inactive OGT variants. Most of the currently known OGT-CDG variants similarly disrupt O-GlcNAc homeostasis, whereas the Genome Aggregation Database (GNOMAD) non-pathogenic OGT variants do not. This approach was then used to dissect new putative OGT-CDG variants from pathogenic background variants in other disease-causing genes. This work enables prediction of pathogenicity for a rapidly growing body of *de novo* OGT-CDG variants and points to changes in O-GlcNAc homeostasis as a common mechanism underpinning OGT-CDG.

(P-127) Modular Economical One-Pot Multienzyme Synthesis of Complex Glycans

Chin Huang[1,2], Kelley W. Moremen[1,2]

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

Glycans are ubiquitous molecules that play essential roles in diverse biological functions and have broad applications, including supplementation of human milk oligosaccharides to infant formula, design of glycan-based vaccines, and functions of homogeneous glycoproteins. Common synthetic methods have challenges in affordable scalability, hindering the development of glycan therapeutic applications. One-pot multienzyme (OPME) systems have streamlined glycan synthesis; yet few have focused on their optimization and scalability. In our work, we succeed in using a stable, affordable phosphate energy source to recycle expensive nucleotide sugars *in situ* for complex glycans synthesis. Given the acidic and cation-sequestering nature of phosphate donors and the large numbers of components in the enzymatic reactions, cross-interactions have been assessed. We established an efficient workflow to rapidly monitor reaction substrates and products at multiple time points by mass spectrometry using an in-house program to analyze and visualize numerous mass spectra. Here, we demonstrated our workflow by adding β1,3-*N*-acetylglucosamine to lactose, starting from inexpensive precursors with catalytic amounts of nucleotides. We have expanded and modularized our economical OPME system using different sugars to synthesize a variety of glycan linkages. Our use of lower nucleotide concentrations, higher sugar input, no intermediate glycan purification, and nearly 100% reaction efficiency pave the path to large-scale synthesis of complex glycans in an ordinary lab setup. Our future goals are to further increase the total turnover number of both nucleotides and enzymes, and to extend our system to plant and bacteria-specific sugars. Facile accessibility to complex glycans can facilitate research on their functions and applications that improve human health.

(P-128) Ashwell-Morell Receptor Ligand Selectivity Encompasses Bi-Antennary N-Glycans in the Absence of a2-3 and a2-6 Sialic Acid Linkages

John Hintze[1,2], 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,*

The Ashwell-Morell receptor (AMR) is also known as the hepatic asialoglycoprotein receptor. The AMR is the prototypical mammalian lectin and the first cell receptor isolated. Endocytic AMR expression and function in the liver determine the half-lives of various blood proteins as they age in circulation. In addition, this clearance mechanism rapidly controls the host blood proteome and is targeted by pathogens that induce neuraminidase activity in syndromes including colitis and sepsis. The AMR thereby plays important roles in both normal physiology and disease. Over 90% of nascent circulating blood proteins are N-glycoproteins bearing one or more N-glycan structures, with over 80% of N-glycan structures being bi-antennary forms. Previous studies comparing AMR ligand specificity have used synthetic neoglycoconjugate ligands and have reported that binding is selective for de-sialylated N-glycans bearing tri-antennary or higher-branched structures with little to no binding to bi-antennary forms. Separately, studies by others have reported that the AMR is capable of binding to sialylated glycoproteins bearing α2-6 linked sialic acid. To address these unexpected findings, we have investigated AMR ligand selectivity using two known AMR N-glycoprotein ligands, namely the IAP and TNAP alkaline phosphatase isozymes. Recombinant IAP and TNAP were produced in glycoengineered CHO cells as fully N-glycosylated selectively with bi-antennary N-glycans capped with either α2-3 or α2-6 linkages of sialic acid (Neu5Ac) or de-sialylated to expose underlying galactose. We have compared circulatory half-lives of both sialylated and de-sialylated glycoforms and have further analyzed their binding to the AMR. Our findings using this approach with native recombinant glycoengineered mammalian N-glycoproteins are distinct from those reported in past publications of

other laboratories. In understanding the source of these differences, we will present our revision to AMR ligand selectivity in the binding and endocytic clearance of native conformations of circulating N-glycoproteins.

(P-129) From Facial Features to Genetic Signatures: Exploring OGT-CDG Phenotypes

Iria Esperon Abril[1], Florence Authier[1], Jesper Skovhus Thomsen[2], Daan van Aalten[1]

[1]*Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark,* [2]*Department of Biomedicine, Aarhus University, Aarhus, Denmark,*

Intellectual disability (ID) is a neurodevelopmental condition that can arise from various genetic causes, including Congenital Disorder of Glycosylation (CDG). A specific variant, OGT-CDG, results from defects in the O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) enzyme. The molecular mechanisms underlying OGT-CDG, including potential disruptions in O-GlcNAc homeostasis, HCF1 processing, or protein misfolding, are not yet fully understood. This syndrome is associated with ID and distinctive craniofacial abnormalities, such as microcephaly and dolichocephaly, along with other dysmorphic features. The primary goal of this study is to characterize the skull and skeletal phenotypes associated with OGT-CDG variants using geometric morphometrics. Preliminary analyses using micro-computed tomography (micro-CT) on mouse models reveal significant craniofacial differences between wild-type and mutant specimens, including notable flattening of skull features and a reduction in endocast volume. These initial findings suggest potential diagnostic markers and offer insights into the structural impact of OGT mutations. Comparative studies with other intellectual disability syndromes and broader skeletal analyses will deepen our understanding of the condition and help identify the genetic and molecular bases of its phenotypes. Further research will focus on investigating the underlying biological pathways and exploring potential therapeutic approaches.

(P-130) Deletion of *Cosmc* (*C1GalT1C1*) in Murine Hepatocytes leads to Tn antigen (CD175) Expression and Altered LDL-Receptor Glycosylation

Rajindra P. Aryal[1], Maxence Noel[1], Junwei Zeng[1], Yasuyuki Matsumoto[1], Rachael Sinard[1], Hannah Waki[1], Florian Erger[2,3], Björn Reusch[2,3], Bodo B. Beck[2,3], Richard D. Cummings[1]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA,* [2]*Institute of Human Genetics, University Hospital Cologne, Faculty of Medicine, University of Cologne, Cologne, Germany,* [3]*Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany,*

Hepatocytes play critical roles in many cellular functions, including metabolism, detoxification, and protein biosynthesis. They produce a vast number of glycoproteins present in their membranes and secretions, many of which contain extended normal O-glycans linked to Ser/Thr residues. However, the role of extended O-glycans on hepatocytes, as well as their secreted glycoproteins in the blood, is not clear. To address this, we generated a targeted deletion of *Cosmc* (*C1Galt1c1*) specifically in murine hepatocytes (HEP-*Cosmc*-KO). Analysis of the liver lysates from the HEP-*Cosmc*-KO mice, revealed that liver glycoproteins specifically lack extended O-glycans and express the Tn antigen (CD175) (GalNAcα1-O-Ser/Thr). Consistent with this finding, we also found a remarkable reduction of T-synthase enzyme activity compared to WT. In the serum from HEP-*Cosmc*-KO mice, we also observed Tn antigen containing glycoproteins. By characterizing the LDL-receptor (LDLR), a heavily O-glycosylated glycoprotein in hepatocytes, we observed a reduction in its molecular weight from ~145kD to around 120kDa in HEP-*Cosmc*-KO mice lysate. Interestingly, we observed no significant differences in the expression level of the LDLR, as well as HMG-CoA reductase, which is typically altered in response to aberrant cholesterol metabolism; overall, the results indicate no significant impact of *Cosmc* deletion on LDLR stability and cholesterol regulation. Along the same line, we did not observe any significant changes in both WT and HEP-*Cosmc*-KO mice phenotype, development, and aging compared to WT. Together, these findings provided new insight into the role of extended O-glycans in liver function and mice development.

(P-131) Exploring the potential of an antimicrobial peptide to direct the evolution of a bacterial glycosyltransferase

Lisa Parsons[1], Willie Vann[1]

[1]*Food and Drug Administration, Silver Spring, MD, USA,*

The bacterial N-glycosyltransferase from *Actinobacillus pleuropneumoniae* (apNGT) was shown by Aebi et al. [2011 *JBC*. 286(4):35267] to transfer glucose from UDP-glucose to NxS/T, the same sequence utilized for N-linked glycosylation in

eukaryotes. While potentially very useful for constructing glycoconjugates, the enzyme has low activity and tolerates only a limited subset of amino acids before, after, and in the middle of the NxS/T sequence [2017 *JBC*. 292(21):8856–8863]. Mutations improving the rate and promiscuity of the enzyme have been identified using sequence and structure-guided approaches [2017 *JBC*. 292(21):8856–8863, 2024 *Engineering Microbiology*. 4(1):100134], but such methods can only explore a relatively small range of mutants due to the necessity to clone and purify each mutant protein prior to screening. Random mutagenesis followed by survival of only mutants with enhanced function would enable screening of a large library of mutants; however, bacterial glycosylation isn't normally required for survival, and thus doesn't lend itself to this high-throughput selection method. Antimicrobial peptides (Apeps) provide a possible solution to this conundrum not just for apNGT, but other glycosyltransferases and glycosidases as well. Apeps are small peptides ranging from 12–50 amino acids long that kill or inhibit bacterial growth, some by binding to high-affinity targets. Addition or subtraction of bulky sugar moieties to these small peptides has the potential to affect the structure and therefore the antimicrobial properties. Here we describe our initial exploration into the potential of buforin II to be modified by and serve as a selective agent for directed evolution of apNGT.

(P-132) Determining Novel Tissue Specific Regulators of O-mannosylation

David E. Steen Jr[1], Zheng Zhang[2], Terrell Carter[1], Xiaolin Dong[1], Erin Suh[1], Ryan Weiss[1], Pengpeng Bi[2], Lance Wells[1]

[1]*Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, 30605, United States of America,* [2]*Department of Genetics, Center for Molecular Medicine, University of Georgia, Athens, Georgia, 30605, United States of America,*

A group of approximately 12 enzymes work together to add an extended O-Mannose structure (referred to as the functional M3 glycan) on the protein alpha-dystroglycan (a-DG) that terminates in a repeating disaccharide motif referred to as matriglycan. Defects in any of the M3 enzymes result in hypoglycosylation of a-DG and are causal for a family of congenital muscular dystrophies referred to as dystroglycanopathies. These degenerative skeletal muscle diseases can also lead to devastating brain and eye abnormalities along with very short lifespans in the most severe forms. Despite multiple gene products assigned as causal for dystroglycanopathies, a purported 30% of cases are still of unknown genetic etiology. Here we present a CRISPR-Cas9 based genetic screen aimed at identifying novel gene products involved in the synthesis and regulation of the functional M3 glycan in an effort to elucidate genes that are causal for these unexplained dystroglycanopathies. We performed the genetic screen in a novel myoblast cell line that can differentiate to myocytes but is incapable of fusion. This cell line provides us, for the first time, the opportunity to Cas9 screen myocytes, that have functional a-DG unlike myoblasts, via MACS and/or FACS sorting of single cells. Utilizing the clinical antibody IIH6, which specifically recognizes matriglycan, we are able to sort cells that have lost a gene that prevents them from producing the functional M3 glycan on cell surface a-DG. We have sequenced these genes and will functionally characterize the novel gene products elucidated from the screen. Further screens in other disease relevant cell lines such as neural cell lines will be carried out. Finally the characterized gene list will be evaluated by our clinical collaborators to potentially explain undefined cases that have the potential to lead to novel avenues of treatment.

(P-133) Cell-based Mucin Array for Discovery and Characterization of Mucinases/O-glycanases and Carbohydrate Binding Modules

Yoshiki Narimatsu[1,2], Thapakorn Jaroentomeechai[1], Felix Goerdeler[1], Ramon Hurtado-Guerrero[1], Henrik Clausen[1]

[1]*Copenhagen Center for Glycomics, Departments of Cellular and Molecular Medicine and School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Denmark,* [2]*GlycoDisplay ApS, Copenhagen, Denmark,*

Mucins and mucin-like proteins contain densely O-glycosylated domains that for long have been unapproachable for detailed structural studies due to their dense O-glycans and heterogeneity. We sought to capture the biological information contained in these dense O-glycodomains from human mucins and mucin-like O-glycoproteins and enable dissection of and the molecular "cues" encoded. To this end we developed a cell-based array platform for the display and production of O-glycodomain reporters with defined O-glycans using stable genetic glycoengineered[1]. Display of the O-glycodomain reporters on the cell surface serves as a facile way to screen and dissect binding interactions by flow cytometry. We have used the cell-based array to probe and dissect the binding specificities of a variety of glycan-binding proteins (GBPs), including antibodies, innate lectins, microbial adhesins, and viruses. A major power of the cell-based array platform is that this also enables the production of secreted O-glycodomains with homogenous glycans for wider studies of interactions with GBPs. We will illustrate this with characterization and discovery of mucin-degrading enzymes (mucinases/O-glycoproteases) [2] as well as mucin-binding modules (MBMs)[1], which do not bind simple oligosaccharides but clustered glycan motifs.

References

Nason R, Büll C, Konstantinidi A, Sun L, Ye Z, Halim A, Du W, Sørensen DM, Durbesson F, Furukawa S, et al. Display of the human mucinome with defined O-glycans by gene engineered cells. *Nat Commun*. 2021:**12**(1):4070.

Narimatsu Y, Büll C, Taleb V, Liao Q, Compañón I, Sánchez-Navarro D, Durbesson F, Vincentelli R, Corzana F, Rovira C, et al. A Family of Di-Glutamate Mucin Degrading Enzymes that Bridges Glycan Hydrolases and Peptidases. *Nat Catalysis*. 2024.

Session 6: Gaining Insights into Glycoenzymes and Glycoconjugates with: Structural Biology

(Key6-001) Neutrons for structural glycobiology in host-pathogen interactions

Anne Imberty[1]

[1]*CERMAV, CNRS, Univ. Grenoble Alpes, Grenoble, France,*

Lectins are often involved in the first steps of the infection process. In many cases, lectins expressed by pathogenic microbes have the ability to specifically recognize complex carbohydrates present on the host tissue. Fucose, sialic acids and galactose are the targets for the lectins of several pathogenic bacteria and viruses. Neutron crystallography is less common than x-ray crystallography but is it a powerful method for localizing hydrogen bonds that play a crucial role in protein-carbohydrate interactions. However, this method requires the production of deuterated biomolecules, which is not trivial for glycans. Perdeuterated monosaccharides and oligosaccharides are produced using a synthetic glycobiology approach and co-crystallized with lectins from Pseudomonas aeruginosa. Diffraction data at Institut Laue Langevin has yielded the first structures of perdeuteurated lectin/carbohydrate complexes, with new finding in the fine mechanisms of recognition.

(Key6-002) Breaking Down the Bottlebrush: Atomically-Detailed Structural Dynamics of Mucins through Molecular Dynamics Simulation

Fiona L. Kearns[1,†], Mia A. Rosenfeld[2,†], Rommie E. Amaro[1]

[1]*Department of Molecular Biology, University of California San Diego, La Jolla, California 92093-0340, United States,* [2]*National Heart, Lung & Blood Institute, National Institute of Health, Bethesda, Maryland 20892, United States,*

[†]These authors contributed equally

Mapping the dynamical features of mucins within the glycocalyx remains a vast and promising frontier as there remain many challenges associated with their structural characterization. Using limited mass spectrometry glycoproteomics and protein sequencing data, we have constructed first-of-their-kind all-atom, explicitly solvated, molecular dynamics (MD) simulations of respiratory mucin models, particularly MUC5B. From our simulations, we describe the key forces and degrees of freedom limitations imposed by extensive O-glycosylation, which thereby imbue the canonically observed "bottlebrush" structure to these otherwise intrinsically disordered protein backbones. We compare our simulation results to static structures observed in recent experimental scanning tunneling microscopy work by Anggara et al. and notice striking resemblance. Our work presents a novel framework for investigating the dynamics and interactions of mucins, while retaining structural details currently inaccessible to experimental techniques.

(Key6-003) A mucin-type O-glycosyltransferase from the protozoan pathogen *Toxoplasma gondii* is distinct from its host homologues

Pranav Kumar[1], Tadakimi Tomita[2], Thomas A. Gerken[3], Collin J. Ballard[3], Yong Sok Lee[4], Louis M. Weiss[2,5], Nadine L. Samara[1,*]

[1]*Structural Biochemistry Unit, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, 20892, USA,* [2]*Department 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]*Department of Medicine (Infectious Disease), Albert Einstein College of Medicine, Bronx 1300 Morris Park Avenue, New York, 10461, USA,*

Mucin-type O-glycosyltransferases (GalNAc-Ts) catalyze the attachment of N-acetylgalactosamine (GalNAc) to Thr or Ser residues on protein substrates to initiate mucin-type O-glycosylation, an abundant and complex post-translational modification that regulates protein stability, structure, and function. In *Toxoplasma gondii*, bradyzoite tissue cysts residing in the CNS or muscle tissue are encapsulated by a glycosylated cyst wall that is critical for immune evasion, survival, and transmission. O-glycosylation of the cyst wall protein CST1 by the mucin-type O-glycosyltransferase *T. gondii* (Txg) GalNAc-T3 influences cyst wall rigidity and stability. A low sequence similarity between TxgGalNAc-T3 and its metazoan homologues hints at a divergence in function, suggesting that specifically targeting TxgGalNAc-T3 could weaken bradyzoites that have thus far been resistant to therapies. X-ray crystal structures of TxgGalNAc-T3 alone and in complex with cyst wall peptides reveal multiple features that are strictly conserved among its apicomplexan homologues, including a unique 2nd metal that is coupled to substrate binding and enzymatic activity in vitro and cyst wall O-glycosylation in *T. gondii*. The study illustrates the divergence of pathogenic protozoan GalNAc-Ts from their host homologues and lays the groundwork for studying apicomplexan GalNAc-Ts as therapeutic targets in disease.

(Key6-004) Glycosyltransferase Bump-and-hole Engineering to Study Proteoglycan Biosynthesis in Mammalian Cells

Zhen Li[1,2], Lucia Di Vagno[2,†], Himanshi Chawla[1,2,†], Aisling Ni Cheallaigh[3], Meg Critcher[4], Douglas Sammon[1], David C. Briggs[2], Nara Chung[5], Vincent Chang[5], Keira E. Mahoney[5], Anna Cioce[1,2], Lloyd D. Murphy[6], Yen-Hsi Chen[7], Yoshiki Narimatsu[7], Rebecca L. Miller[7], Lianne I. Willems[6], Stacy A. Malaker[5], Mia L. Huang[4], Gavin J. Miller[3], Erhard Hohenester[1], Benjamin Schumann[1,2]

[1]*Imperial College London,* [2]*The Francis Crick Institute,* [3]*Keele University,* [4]*Scripps Research,* [5]*Yale University,* [6]*University of York,* [7]*University of Copenhagen,*

[†]Equal contribution

Glycosaminoglycans (GAGs) are major determinants of proteoglycan function and contribute substantially to the cell-surface glyco-code. While fully-established GAG chains are structurally highly variable, attachment to the core protein is manifested through a conserved tetrasaccharide linker. The first peptide-proximal monosaccharide is xylose, linked through an O-glycosidic bond predominantly to Ser residues by protein xylosyltransferase (XylT) enzymes. Human cells express the two homologous isoenzymes XylT1 and XylT2 that are individually associated with disease reflecting dysfunctional proteoglycans. Yet, the molecular details underpinning the functions of both isoenzymes are ill-defined. Here, we develop a chemical biology approach *en route* to dissect the protein substrate specificities of human XylTs. In a structure-based tactic termed bump-and-hole engineering, we mutate XylTs to incorporate a chemically modified analogue of the natural substrate UDP-xylose that is not accepted by wildtype enzymes. We characterize the kinetic properties of the bump-and-hole system and show that engineered XylT1 retains the peptide substrate specificity of the WT enzyme. We develop a strategy to biosynthesize the UDP-sugar and establish cellular bump-and-hole systems for both XylT1 and XylT2 that allow chemical profiling and manipulation of proteoglycans. Our precision tools will provide insight into the repertoire and biosynthetic details of proteoglycans.

(PT-010) Structural and functional analysis of mannosyltransferases responsible for high-mannose *N*-linked glycan synthesis in the endoplasmic reticulum

J. Andrew N. Alexander[1], Shu-Yu Chen[2], Somnath Mukherjee[3], Mario de Capitani[4], Rossitza N. Irobalieva[1], Lorenzo Rossi[1], Markus Aebi[5], Jean-Louis Reymond[4], Anthony A. Kossiakoff[3], Sereina Riniker[2], Kaspar P. Locher[1]

[1]*Institute of Molecular Biology and Biophysics, Eidgenössische Technische Hochschule (ETH) Zürich, Zürich, Switzerland,* [2]*Department of Chemistry and Applied Biosciences, Eidgenössische Technische Hochschule (ETH) Zürich, Zürich, Switzerland,* [3]*Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, USA,* [4]*Department of Chemistry and Biochemistry, University of Bern, Bern, Switzerland,* [5]*Institute of Microbiology, Eidgenössische Technische Hochschule (ETH) Zürich, Zürich, Switzerland,*

N-linked protein glycosylation in eukaryotes is associated with many processes of relevance for biology and biomedicine. Assembly of *N*-linked glycans is initiated by the Asparagine-Linked Glycosylation (ALG) enzymes, which transfer monosaccharides to a growing dolichol-linked oligosaccharide on both sides of the endoplasmic reticulum membrane. Here we investigated the structure and function of the three integral membrane mannosyltransferases responsible for producing high-mannose *N*-glycans in the endoplasmic reticulum lumen: ALG3, ALG9, and ALG12. These class-C_A glycosyltransferases extend dolichol-PP-$GlcNAc_2Man_5$ to dolichol-PP-$GlcNAc_2Man_9$, creating the B- and C-branches of the *N*-glycan. ALG3 initiates the B-branch

with the addition of an α1,3-linked mannose while ALG12 begins the C-branch with an α1,6-linked mannose. ALG9 concludes the B- and C-branches by adding α1,2-linked mannoses. While mutations in these ALG enzymes are linked to congenital disorders of glycosylation (CDG), their structures as well as mechanisms of substrate selection and catalysis have remained elusive.

Using cryo-EM, we determined high-resolution structures of ALG3, ALG9, and ALG12 in complex with chemoenzymatically synthesized donor and acceptor substrates. By visualizing four ternary complexes capturing states prior to mannose transfer, we identify the substrate binding sites and obtain insight into their mechanisms of catalysis. Our structures, combined with functional assays and molecular dynamics simulations, provide a molecular understanding of how acceptor and donor substrate specificity is controlled and why mannose addition occurs in a strictly ordered manner during biosynthesis. The ALG3 ternary structure shows that the active site can only accommodate acceptor substrates lacking a C-branch, providing an explanation for why ALG12 only acts on substrates already processed by ALG3 *in vivo*. The donor substrate Dol-P-Man is bound to ALG3 with the sugar poised for transfer in a bent-back conformation. The ternary structures of ALG9, containing either Dol-PP-$GlcNAc_2Man_6$ or Dol-PP-$GlcNAc_2Man_8$, demonstrate how two different acceptor substrates are accommodated by the same enzyme. Molecular dynamics simulations with ALG9 and Dol-P-Man, Dol-P-Glc, or Dol-P-2-fluoro-Man suggest how dolichol-P-Man is selected as the donor substrate from the ER membrane over its C2 epimer, dolichol-P-Glc. The ALG12 ternary structure reveals an acceptor B-branch recognition pocket, providing a molecular basis for selecting the dolichol-PP-$GlcNAc_2Man_7$ substrate over earlier pathway intermediates that lack a completed B-branch. Our studies provide novel insight into the *N*-linked glycosylation pathway, with implications for understanding CDGs and facilitating future biotechnological applications.

(PT-012) Computational Insights into the Modulation of N-glycosylation on the Pentameric Assembly and Channel Gating of $GABA_A$ Receptors

Amin Akbari Ahangar[1], Jing Li[1]

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

$GABA_A$ receptors ($GABA_A$Rs), essential for rapid synaptic transmission in the central nervous system, are pentameric ligand-gated ion channels, typically in the (alpha X alpha XX) configuration. The molecular determinant of this selective pattern has remained a decade-long mystery. These proteins undergo extensive N-linked glycosylation, both on the protein surface and central pore. These glycans regulate channel function and are implicated in neurological diseases such as epilepsy and schizophrenia. Despite their importance, the structural basis of glycosylation impacts on channel function remains unknown. We hypothesize that pore-facing glycans regulate channel assembly by preventing the formation of unfavorable pentamers, while surface glycans stabilize the GABA binding. Using microsecond-scale molecular dynamics simulations, we examined the impact of glycosylation on $GABA_A$R pentamers. The models with two (wild-type) and more than two pore-facing glycans, along with different populations of surface glycans, were simulated and analyzed. Our findings indicate that pore-facing glycans significantly influence $GABA_A$R pentamer stability and permeability. The presence of two glycans maintains the extracellular domain's diameter, supporting channel functionality. In contrast, models with more than two glycans exhibited expansion, leading to the closure of a hydrophobic gate and impaired channel function. Moreover, surface glycans protect the loop C through hydrogen bonds while limiting subunit rearrangement through interaction with adjacent subunits. Overall, proteins with surface glycans exhibit more stable GABA binding. This research elucidates the pivotal role of glycosylation in $GABA_A$Rs function, offering insights into therapeutic strategies targeting neurological disorders associated with $GABA_A$Rs dysfunction.

(PT-014) Development of a rhamnogalacturonan II (RG-II) glycoform libraryto study identify the sequence-structure determinants of borate diester formation

Deepak Sharma[1], Sabina Koj[1], Kristen Thorne[1], Liang Zhang[1], John Glushka[1], Stephanie Archer-Hartmann[1], Ian M. Black[1], Vivek Bharadwaj[3], Micheal Crowley[3], Lintao Bu[3], Malcolm A. O'Neill[1], Breeanna R. Urbanowicz[1,2]

[1]*Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, USA,* [2]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA,* [3]*Renewable Resources and Enabling Sciences Center, National Renewable Energy Laboratory, Golden, CO, USA,*

Rhamnogalacturonan-II (RG-II) is a domain of pectin that exists as a borate cross-linked dimer in the cell walls of all vascular plants. Formation of this dimer is necessary for plant growth and development. Results of previous studies indicated that the glycosyl sequence of RG-II has an important role in controlling the rate of dimer formation and the regiospecifity of the crosslinking reaction. To substantiate this claim, we used *Bacteroides thetaiotaomicron* glycoside hydrolases to generate RG-II glycoforms with structurally defined A and B sidechains. In vitro dimerization assays showed that several modifications of the glycosyl sequence of RG-II significantly changed its propensity to dimerize. The rate of dimer formation increased when methyl

fucose (MeFuc) was removed from chain B (RG-II$^{B\Delta MeFuc}$). In contrast, it was markedly slowed by in glycoforms lacking terminal l-galactose (l-Gal) from chain A(RG-II$^{A\Delta Gal}$). Generation of RG-II$^{B\Delta MeFuc\text{-}A\Delta Gal}$ double variants restored the dimerization rate to near normal. We hypothesized that these changes in dimer propensity can be explained by the changes in the chemical environment around the apiose responsible for crosslinking with borate, which is supported by combined HETCOR NMR and molecular modelling studies. Taken together, our data suggests that the core glycosyl sequence of RG-II is conserved to enable it to adopt conformations that regulate the rate at which the dimer forms and to prevent the formation of dysfunctional crosslinks.

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(PT-016) Interbacterial warfare in plant colonization: structural biology to understand recognition of glycan receptors by tailocin tailfiber proteins

Martin P. Horvath[*,1], Lauren Winn[1], Genesis Michelle Viquez Pinzon[1], Talia Backman[1], Talia L. Karasov[1]

[1]*University of Utah, School of Biological Sciences, 257 S 1400 E, Salt Lake City, Utah 84112, USA,*

The many microbial species that colonize plants compete with one another on the plant. To win this battle, pathogenic plant microbes make use of molecular killing machines that resemble the tail apparatus of bacteriophages and are therefore called tailocins. Tailocins exhibit high specificity in their killing - the tailocins made and released through lysis by a plant pathogen do not attack plant cells or structures, instead kill a subset of other competing bacteria, and somehow avoid killing bacteria of the same strain. Killing specificity appears to depend on a particular glycan receptor built into the lipopolysaccharide (LPS) component that is found attached to the outer membrane of susceptible bacteria, but which is missing or otherwise hidden for resistant bacteria. The glycan receptor binding domains are thought to be located at the tips of tailfiber proteins, long trimeric proteins that extend from the baseplates of a tailocin, with six such tailfibers per tailocin. We know the sequences for several different tailfiber proteins from naturally occurring *Pseudomonas viridiflava* strains, and find that these are evolving rapidly, especially in the very C-terminal portion, which corresponds with the predicted glycan receptor-binding domain. We are pursuing two complementary approaches to understand molecular recognition for glycan receptor-binding proteins and their target glycan receptors. In one strategy we aim to crystallize the proteins and determine structures through x-ray crystallography. Toward this goal we have developed expression systems for both endogenous expression in *Pseudomonas* and heterologous expression in *E. coli*. In a complementary strategy we are screening potential glycan receptors virtually, docking these with the structures of tailfiber proteins predicted by ColabFold. Ultimately we hope to discover the structural basis for strain-specific suppression of a common pathogen in plants, with far-reaching implications for food security and control of antibiotic-resistant bacteria.

(P-134) A non-prolyl cis peptide bond plays an essential role in the high binding affinity to oligomannose *N*-glycans of specific Endoglycosidases

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

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA,*

High affinity is the most critical parameter for biomolecular recognition and interaction of the biomolecules. Recently, we described a bacterial endoglycosidase (ENGase) from *Bacteroides thetaiotaomicron* showing unusually high affinity to High-Mannose (HM) *N*-glycan substrates. Applying a directed evolution approach, we generated inactive ENGase variants with increased affinity for the HM substrate, one of which exhibited a 70-fold affinity increase from ~3nM to ~40pM to HM *N*-glycans on human IgGs as determined by SPR analysis. This variant has mutations in residues forming a non-prolyl cis peptide bond located close to the active site in the TIM barrel structure of the enzyme. Non-prolyl cis peptide bonds are extremely rare in protein structures but nearly all genuine cis peptide bonds of this nature play an important role in molecular function. Here, we used a combination of biochemical and structural approaches to determine the essential role of this unconventional cis peptide bond for ENGases to bind and hydrolyze HM *N*-glycan substrates. Our findings could be useful for specifically targeting HM glycoproteins *in vitro* and *in vivo* assays for biomedical and or biotechnological purposes.

(P-135) Computational Mutagenesis of Lamprey Antibodies to Enhance Specificity for Sulfated Glycans

Zhe Yang[1], Maxence Noel[2], Ea Tulin[2], Richard D. Cummings[2,*], Robert J. Woods[1,*]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA,* [2]*Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA*

Sulfation is a common and significant post-translational modification in various biological molecules, playing a crucial role in numerous biological functions. Sulfated glycans, in particular, are linked to several diseases and hold significant promise in molecular pathology as biomarkers. However, the isolation and detection of sulfated glycans is challenging. This study aims to apply computational methods to guide the mutagenesis of Lamprey antibodies, enhancing their specificity for sulfated glycans that are known to be prevalent in brain tissue. These new reagents will help provide deeper insight into the biological roles of these complex molecules.

First, the Lamprey antibody-disaccharide (Gal1-4GlcNAc-OH) complex will undergo fully solvated molecular dynamics (MD) simulation using the AMBER/GLYCAM force field for proteins and carbohydrates. The stability of the MD trajectories will be evaluated by monitoring the root-mean-squared deviation (RMSD) of the protein Cα atomic positions and the disaccharide ligand atoms. Starting from the final conformation of the previous MD simulation, the sulfated disaccharide (Gal[3S][6S]1-4GlcNAc[6S]-OH) will then undergo additional MD simulation with a minor restraint to stabilize it in the binding site. This setup will enable future calculations of per-residue contributions and alanine scanning using the molecular-mechanics generalized Born solvent approximation (MM-GBSA).

To achieve Lamprey antibodies specificity for sulfated glycans, alanine scanning is crucial. Beyond traditional alanine scanning, we leverage advanced modeling techniques to design modified alanine residues with specific charges: one variant with a positive charge (positive alanine) and another with a negative charge (negative alanine). By running traditional alanine scanning, positive alanine scanning and negative alanine scanning on multiple combinations of sulfated protein-ligand complexes, we can thoroughly explore the effects of repulsion and attraction on protein-ligand interactions. This innovative approach allows for a detailed comparison of these interactions, enabling us to determine how specific mutations in antibody residues can enhance attractive or repulsive forces. Ultimately, this fine-tuning of the specificity for different sulfated disaccharides will provide deeper insights and more effective tools for studying the biological roles of these complex molecules.

(P-136) Impact of the α2,3-sialyl N-glycosylated prostate-specific antigen (PSA) as a biomarker for prostate cancer in men with gray zone levels of the PSA

Yuki Miura[1], Shingo Hatakeyama[1], Tohru Yoneyama[1], Naoki Fujita[1], Teppei Okamoto[1], Hayato Yamamoto[1], Chikara Ohyama[1]

[1]*Hirosaki University School of Medicine, Japan,*

Introduction and Objective: The presence of glycosylated isoforms of the prostate-specific antigen (PSA) in prostate cancer (PC) cells is a potential marker of the aggressiveness of PC cells. This study aimed to characterize the origin of the α2,3-sialylated PSA (S23PSA) using tissue-based sialylation-related gene expression and assess the performance of S23PSA alone and in combination with multiparametric magnetic resonance imaging (mpMRI) to detect PC in men with grey zone levels of PSA.
Methods: We correlated S23PSA with Prostate Imaging Reporting and Data System (PI-RADS) scores in 149 men enrolled in a single-center MRI-targeted biopsy cohort. We compared the diagnostic performances of S23PSA, PI-RADS scores, and total PSA (tPSA) for PC. Further, we investigated the diagnostic performance of S23PSA combined with tPSA with or without PI-RADS in men with grey zone levels of PSA (4–10 ng/mL).
Results: S23PSA was superior to tPSA (area under the receiver operating characteristic curve [AUC]: 0.741 vs. 0.571) but equivalent to PI-RAD scores (AUC: 0.757). In grey zone levels of PSA, S23PSA + PI-RADS + tPSA was superior to PI-RADS + tPSA with avoidance rate of MRI–TBx (18,7% vs. 15,2%) at 57% risk threshold.
Conclusions: The diagnostic performance of S23PSA was superior to conventional strategies in grey zone levels of PSA.

(P-137) Structural studies of glucosyltransferase ALG10 bound to chemo-enzymatically generated substrate analogs

Lorenzo Rossi[1], Mario de Capitani[2], Somnath Mukherjee[3], Julia Kowal[1], J. Andrew N. Alexander[1], Anthony A. Kossiakoff[3], Jean-Louis Reymond[2], Kaspar P. Locher[1]

[1]Institute of Molecular Biology and Biophysics, ETH Zürich, Zürich, Switzerland, [2]Department of Chemistry and Biochemistry, University of Bern, Bern, Switzerland [3]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA,

N-linked glycosylation is an essential modification that affects a multitude of biological functions. In eukaryotes, this process is initiated in the endoplasmic reticulum and involves several ALG (asparagine-linked glycosylation) enzymes, which utilize dolichyl-phosphate activated sugars to synthesize the lipid-linked oligosaccharide (LLO) donor to be transferred to acceptor proteins by the oligosaccharyltransferase. The last enzyme in the LLO biosynthesis pathway is the integral membrane protein ALG10, a C_A-class glycosyltransferase which adds the terminal α-1,2 glucose to the A-branch of the LLO. Mutations of this enzyme have been implicated in severe hypoglycosylation defects, human congenital disorders of glycosylation and colorectal cancer. In the absence of structural insight, the catalytic mechanism of ALG10 and the molecular basis of substrate recognition remain poorly understood.

Aided by synthetic Fab fragments, we determined a cryo-electron microscopy structure of nanodisc-reconstituted ALG10 in complex with chemo-enzymatically generated substrate analogs. The EM map reveals the binding sites for both donor and acceptor analogs, rationalizing substrate specificity. The structure further suggests which residues have important functional roles, allowing us to formulate a reaction mechanism. Our interpretations are supported both by evolutionary data and *in vitro* functional assays using wild type and mutant variants of the enzyme. These findings advance our mechanistic understanding of lipid-linked oligosaccharide biosynthesis for protein N-glycosylation and open new directions for the enzymatic synthesis of glycoconjugate therapeutics.

(P-138) The Serendipitous Discovery of Selective Silicate Inhibition of the Mucin Core Elongating Transferases

Collin Ballard[1], Haley Aharoni[1], Matthew Smutny[1], Hana Lee[1], Lam Chou[1], Thomas Gerken[1]

[1]Case Western Reserve University, School of Medicine, Department of Biochemistry, Cleveland, OH,

Mucin type O-glycosylation is a highly complex and essential post translational modification that is found on ~80% of membrane bound and secreted proteins. This process is initiated by a family of 20 GalNAc-Ts which transfer an alpha GalNAc from a UDP-GalNAc donor to the hydroxyl groups of Thr and Ser residues of proteins forming the Tn antigen. This antigen can be further elongated by a variety of Golgi resident glycosyltransferases including the C1GALT1 (Core-1 transferase) forming the core-1 structure (T antigen: Galβ1-3GalNAc-α-O-Ser/Thr), the B3GNT6 (Core-3 transferase) forming the core-3 structure (GlcNAcβ1-3GalNAc-α-O-Ser/Thr), and the ST6GalNAc-I and -II transferases both of which can form the sialyl Tn (STn) antigen (Neu5Acα2-6GalNAc-α-O-Ser/Thr). These transferases and glycan structures have varying but important roles in the progression of many diseases including many cancers making them intriguing targets for drug design. In this work, we have unexpectedly come across an unknown component of sodium silicate that acts as a selective inhibitor against the C1GALT1 and ST6GalNAc-I transferases while having little to no impact on the activities of B3GNT6 and ST6GalNAc-II. Upon heating, the inhibitory effects of the silicate are lost suggesting that the species may be multimeric or cyclic silicates in nature. We further showed that the presence of Arg containing peptides and glycopeptides prevented silicate inhibition presumably due to strong silicate interactions with the guanidino groups of Arg. Kinetic analysis of the C1GALT1 reveals that the silicate species competitively inhibits the binding of substrate and UDP-Gal likely interacting with Arg152 in C1GALT1's catalytic site. Previous studies have shown that Arg152 is important for coordinating UDP-Gal binding and for full C1GALT1 activity (González-Ramírez et al., 2022). An analysis of ST6GalNAc-I's predicted active site reveals Arg residues that may be important for CMP-Neu5Ac binding and may also be targeted by the silicate resulting in its inhibition. Since there are fewer Arg residues in B3GNT6 and ST6GalNAc-II active sites there is little to no inhibition. These results suggest that the presence of Arg residues in substrates and enzymes active sites play important roles in the inhibitory effects of silicates. This study is the first to demonstrate selective silicate inhibition of C1GALT1 and ST6GalNAc-I and provides further details on the mechanisms surrounding these transferases. Additionally, these findings may have implications in the mechanistic roles of C1GALT1 and ST6GalNAc-I in asbestos and silicate derived diseases including mesothelioma and silicosis. Finally, this study reveals the importance of Arg residues in the active sites of C1GALT1 and ST6GalNAc-I that could potentially lead to developing specific inhibitors.

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(P-140) Glycosylation Weakens Skp1 Homodimerization in *Toxoplasma gondii* by Interrupting a Fuzzy Interaction

Donovan A. Cantrell[1], Ramona J. Bieber Urbauer[1,3], Hyun W. Kim[1,2], Robert J. Woods[1,2], Jeffrey L. Urbauer[1,3], Zachary A. Wood[1], Christopher M. West[1,2,4]

[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]*Department of Chemistry, University of Georgia, Athens, GA, USA,* [4]*Center for Tropical and Emerging Diseases, University of Georgia, Athens, GA, USA,*

Skp1/Cullin1/F-Box protein (SCF) complexes represent a major class of E3 ubiquitin ligases responsible for proteomic control throughout eukaryotes. Target specificity is mediated by a large set of F-box proteins (FBPs) which each associate with the Skp1 adaptor via its F-box domain. In the social amoeba *Dictyostelium*, Skp1 is regulated by oxygen-dependent glycosylation which alters Skp1's FBP interactome and inhibits homodimerization involving an ordered interface which partially overlaps with that of FBPs. Based on sedimentation velocity experiments, Skp1 from the intracellular pathogen *Toxoplasma gondii* exhibits a K_d comparable to that of a previously measured FBP/Skp1 interaction. Glycosylation of Skp1's disordered C-terminal region (CTR) distal to the ordered homodimer interface significantly weakens Skp1 homodimerization, an effect reproduced by CTR deletion. Scrambling the CTR retains the high affinity homodimer ruling out an extension of an ordered dimer interface. Replacing the CTR with poly-serine weakens the homodimer to an equal degree as deletion of the CTR, indicating a composition dependent effect. The contribution of the CTR to Skp1 homodimerization is canceled by high salt implicating a role for electrostatics. All atom molecular dynamics simulations with explicit water suggest that the CTR promotes homodimerization via a charge block mechanism. Taken together, the data indicate that glycosylation weakens homodimerization by disrupting a C-terminal fuzzy interaction that functions in tandem with the ordered dimer interface, thereby freeing Skp1 for FBP binding. Thus the CTR contributes to Skp1/Skp1 and Skp1/FBP interactions via independent mechanisms, indicating dual constraints on the evolution of its sequence.

(P-141) Understanding the origin of glycoform diversity as a function of glycoprotein shape

Rajan Kandel[1], Eliza Gazaway[1], Oliver C. Grant[1], Robert J. Woods[*,1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602,*

The posttranslational modification of glycoproteins by N-glycans and their subsequent processing by cellular glycosylation machinery in the ER and Golgi is a profoundly important process that remains largely beyond our ability to manipulate with precision. Interestingly, large variations can be observed in the predominant type of N-glycan present at different glycosylation sites on the same glycoprotein. Differences in glycosite processing levels are critically important, for example, in innate[1] and adaptive[2] immune responses. However, an explanation for the origin of these differences has not emerged. Specifically, in a glycoprotein with multiple glycosites, it is not known how the N-linked glycans at each glycosite can be differentially processed. This is particularly intriguing, given that each glycosite in a glycoprotein is exposed equally spatially and temporally to the same glycan processing enzymes in the ER and Golgi. Here we explore the hypothesis that differential glycosite microheterogeneity arises from differences in the accessibility of the glycans at each site to the processing enzymes, due primarily to variations in the 3D topology of the glycoprotein surface near each glycosite.

To test this idea, 3D models of several glycoproteins, including: Pdi1p, 5'nucleotidase, Erythropoietin and Integrin alpha-5, were generated using molecular dynamics (MD) simulations. The high-mannose form of each glycoprotein was simulated, as it represents the single glycoform that was present at the genesis of the glycoprotein in the ER. The glycans at each glycosite were then assessed for their ability to align into the active site of the first enzyme in the processing cascade (ER Mannosidase I, ERManI) without resulting in a collision between the glycoprotein and ERManI surfaces. This approach was largely able to predict those glycosylation sites that remain unprocessed. As the 3D structural basis for processing is revealed, it enables the rational design of changes that target the degree of processing that occurs at individual N-glycosylation, paving the way towards rational glycoengineering, and providing an important missing piece in the puzzle of protein glycosylation control. We outline the subtleties required to successfully employ the technique and discuss its limitations.

References

Khatri K et al. *Mol. Cell. Proteomics*. 2016:**15**:1895.
Grant OC et al. *Sci. Rep*. 2020:**10**:14991.

(P-142) N-glycosylation: Utility as a Prognostic Biomarker for Renal Cell Carcinoma and Beyond

Caroline Kittrell[1], Lyndsay E.A. Young[1,2], Aaron Angerstein[1], Grace Grimsley[1], Anand S. Mehta[1,2], Tamara Nowling[3], Peggi M. Angel[1,2], Jessica Schmitz[4], Jan Hinrich Braesen[4], Richard R. Drake[1,2]

[1]*Department of Cell and Molecular Pharmacology and Experimental Therapeutics, College of Medicine, Medical University of South Carolina, Charleston, SC, USA,* [2]*Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA* [3]*Division of Rheumatology & Immunology, Medical University of South Carolina, Charleston, SC, USA,* [4]*Nephropathology Division Institute of Pathology, Hannover Medical School, Hannover, Germany,*

Clear cell renal cell carcinoma (ccRCC), one of the most common kidney cancers, is characterized by a distinctive clear cell appearance due to aberrant glucose and lipid metabolism. Surface N- glycans are therefore altered in response to these metabolic permutations. Potentially, changes in the N-glycan signature of renal tissue may serve as a novel source of both diagnostic biomarkers for cancer screening and prognostic biomarkers for response to immunotherapy treatment. In this study, we utilized Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI) to characterize the N-glycome of formalin fixed paraffin embedded renal tissue with ccRCC. Clinical information regarding response to immunotherapy was also analyzed and correlated with the expression levels of two phosphorylated N-glycans. Additionally, we have extensively defined the N-glycome of normal renal structures including glomeruli, proximal convoluted tubules, distal convoluted tubules, medulla and the interface between the renal cortex and medulla. Tubule N-glycans are typified by bisecting multi-fucosylated branched structures distinct from glomeruli. These efforts to develop a normal renal glycan signature may serve in the timely diagnosis of other kidney diseases such as acute kidney injury and lupus nephritis. Multilayered image analysis including immunofluorescence and Mayer's hematoxylin staining were co-registered with N-glycan distribution images to associate specific N-glycans with unique tissue features. Finally, we have incorporated Matrix Assisted Laser Desorption/Ionization Immunohistochemistry (MALDI-IHC), an innovative mass spectrometry imaging approach to begin to delineate the glyco-immune landscape of ccRCC, a cancer often treated with immune checkpoint blockade following metastasis.

(P-143) Valorization of Industrial Hemicellulosic Wastes for Next Generation Biomaterials

Daniel Josey[1], Anthony Heiges[1], Sekhar Yadavalli[1], Breeanna Urbanowicz[1]

[1]*University of Georgia, Athens, GA,*

Polysaccharides from plant biomass are the most abundant biopolymers on Earth, and they represent a hugely underutilized renewable carbon source. While cellulose is already widely used in industry and commercial products, other hemicellulosic oligomers and polysaccharides are almost universally seen as waste products. This is primarily due to their structural heterogeneity and lack of solubility in aqueous and most organic solvents. This work focuses on developing methods to valorize xylan, which composes 25–35% of the dry biomass of woody tissues. Xylan has been oxidized to di-aldehyde xylan (DAX) to add aqueous solubility, and can then be reacted with aliphatic amine to create a potential thermoplastic, or with methacrylate to create an elastic hydrogel. Our future research will further characterize the properties of the DAX-Thermoplastic and DAX-Hydrogel, along with investigating potential commercial applications for the products.

(P-144) Exploring the accuracy of current techniques in glycoprotein modelling

Eliza Gazaway[1], Rajan Kandel[1], Oliver C. Grant[1], Robert J. Woods[*,1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602,*

Covalently linked N-glycans originate as $Glc_3Man_9GlcNAc_2$-Asn, and modification of the glycans occurs as a newly translated glycoprotein is in flux through the endoplasmic reticulum (ER) and Golgi apparatus, being exposed to glycosidases and glycosyltransferases. Varying levels of N-glycan processing are observed across different glycosites on the same glycoprotein, even though each site is exposed to the same enzymes for the same amount of time. We hypothesize that the underlying protein topology sterically prevents enzyme accessibility to glycan substrates at certain sites, resulting in varying levels of processing. Our group is currently developing a computational protocol to predict the extent of N-glycan processing as a function of enzyme accessibility of early modifying enzyme ER mannosidase I (ERmanI) to different conformations of $Man_9GlcNAc_2$ derived from molecular dynamics (MD) on individual N-glycan sites. While the protocol accurately predicts the processing of several glycoprotein systems, predictions for a select few systems are less successful. For example, modelling predicts both N-glycans of CD14 to be high mannose, while LC-MS/MS analysis indicates that site N151 is processed by ERmanI[1]. In the MD simulations,

$Man_9GlcNAc_2$ at site N151 forms extensive hydrogen bonds with the protein surface, making the glycan inaccessible to the 3D structure of ERmanI in the post-simulation analysis. The disagreement between modelling and experiment raises the question of whether conventional molecular dynamics (MD) simulations can accurately model the conformational ensemble of glycans on the surface of the protein. Although MD modelling of glycoproteins has been increasingly used, the accuracy of the predicted conformational ensemble has rarely been validated against experimental data. Here, we compare the modelling ensemble of glycan conformations of solution glycoprotein structures to NOE-derived distance restraints between the N-glycan and the underlying protein. Initial conventional MD simulations of the solution structures fails to produce a conformational ensemble that satisfies all glycan-protein distance restraints, suggesting that conventional MD is unable to sample all biologically relevant low-energy conformations on the μs scale. We further assess whether enhanced sampling MD techniques, including Hamiltonian replica exchange, accelerated MD, and Gaussian Accelerated MD, may be necessary to accurately model the motion of N-glycans in solution.

References

Quintana JI et al. The impact of glycosylation on the structure, function, and interactions of CD14. *Glycobiology.* 2024. https://doi.org/10.1093/glycob/cwae002.

(P-145) Insights into the Biophysical Characterization of Glycopeptide-Cellulose Nanocrystal composite for Application in Food Packaging

Shimona Ahlawat[1,2], Prem Kumar[3], Kulwinder Singh Sran[1,†], Ved Prakash Giri[1], Kamaljit Kaur[3], Vijayakumar Shanmugam[3,§], Alka Rao[1,2,4,‡]

[1]CSIR-Institute of Microbial Technology, Sector 39A, Chandigarh 160036, India, [2]Academy of Scientific and Innovation Research (AcSIR), Ghaziabad 201002, India [3]Institute of Nano Science and Technology, Sector 81, Knowledge City, Mohali, Punjab 140306, India, [4]Present address: Food Safety and Standards Authority of India (FSSAI), New Delhi 110002, India,

[†]First author
[‡]Corresponding author
[§]Co-correspondending author

Food spoilage and contamination pose significant challenges to food safety and preservation. The growing need for sustainable and effective antimicrobial agents in food packaging has led to the exploration of novel composite materials. This study focuses on the development and biophysical characterization of glycopeptide-enriched cellulose nanocrystals (CNCs) as antimicrobial polymeric films designed for food packaging and preservation. Glycocins, potent bacteriocins, were immobilized onto CNCs-PVA solution using the TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) oxidation method, specifically with Glycosylated Enterocin 96 peptide (GEC), which is active against Listeria monocytogenes, a foodborne pathogen. Additional peptides, including Enterocin 96 (EC) and Nisin, were also incorporated into the CNC matrix to compare their antimicrobial effectiveness. Stability assays at different pH levels demonstrated that GEC maintains its activity over a wider pH range compared to Nisin.

To examine the structural and biophysical properties of the CNC-glycocin composite, various analytical techniques were employed. Scanning electron microscopy (SEM) revealed the morphological characteristics, Fourier-transform infrared spectroscopy (FTIR) identified functional groups, X-ray diffraction (XRD) provided insights into crystalline structures, and thermogravimetric analysis (TGA) assessed thermal stability. Dynamic light scattering (DLS) analysis revealed excellent stability, evidenced by a surface potential of –28.6 mV. Surface profiler measurements determined the surface roughness of the films, while contact angle measurements assessed the hydrophobicity and wettability of the composite materials. The antimicrobial activity of the composite was assessed against L. monocytogenes EGD-e serotype 1/2a by diffusion agar assays, which indicated substantial inhibition zones, demonstrating peptide release from the membrane and a reduction in bacterial counts in the CNC-GEC and CNC-Nisin membranes. Overall, CNC-GEC membranes demonstrated the best results as antibacterial agents and can be considered for further studies for food preservation.

(P-146) PROBING O-MANNOSYLATED SITES VIA BIOORTHOGONAL SELECTIVE EXOENZYMATIC LABELLING (SEEL)

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

[1]Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, United States, [2]Department of Chemistry, University of Georgia, Athens, Georgia, United States [3]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, United States, [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 on other proteins, 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, allowing for enrichment of O-mannosylated sites independently of matriglycan. We established utilizing O-Man synthetic peptides and recombinant enzymes that both POMGNT1 and POMGNT2 would utilize UDP-GlcNAz as a donor. Leveraging bioorthogonal selective exoenzymatic labelling (SEEL), O-mannose sites were enriched for on the surface of HEK293 cells. The O-mannose sites were extended by POMGNT1 and POMGNT2, glycosyltransferases that mediate the branching point of the O-mannosylation pathway, and an azido-modified form of UDP-GlcNAc (UDP-GlcNAz). Subsequentially, the azido-sugar modified O-mannosylated sites were clicked with a bioorthogonal biotin tag for neutravidin enrichment. Along with known O-mannosylated proteins α-DG and KIAA1549, adipocyte plasma membrane associated protein (APMAP) and laminin subunit beta 1 (LAMB1) were also enriched and identified as potentially novel O-mannosylated proteins. We are currently evaluating whether either of these proteins are O-mannosylated or whether they might interact tightly with α-DG. Furthermore, we are conducting the SEEL experiment in more disease relevant cell lines including myocytes and neurons in order to identify if other O-Mannosylated glycoproteins might indeed be partially extended via the M3 pathway and potentially contribute to the disease phenotype.

(P-147) A general strategy to characterize structures of polysaccharides by molecular dynamics incorporating short-range NMR information

Hugo F. Azurmendi[1], Audra A. Hargett[1], Conor J. Crawford[2,3], Maggie P. Wear[2], Stefan Oscarson[3], Arturo Casadevall[2], Darón I. Freedberg[1]

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

NMR is an excellent tool for characterizing and determining biomolecular solution structures. Characterizing polysaccharides (PS) by NMR, however, is challenging due to the lack of long-range experimental data accessible to researchers. PS consist of a string of repeating units (RU), in turn comprised of glycans numbering from one up to a few units. Because of the repeating nature of chemical environments along the PS, it is rarely possible to obtain long-range NMR information leading to experimentally representative structures. Molecular dynamics (MD) simulations are used to characterize PS structures, but incorporation of crucial experimental input is lacking due to the mentioned problems. In this presentation we describe a promising approach to address these limitations.

Cryptococcus neoformans is a fungal pathogen responsible for cryptococcosis and cryptococcal meningitis. The *C. neoformans* capsular PS and shed exo-PS function both as key virulence factors and to protect the fungal cell from phagocytosis. The PS of a clinically predominant strain, serotype A, is characterized by an RU 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 (the PS is thus referred as GXM), plus *O*-acetylation at the 6 positions of the first and third Man. The smallest unit effectively recognized by anti-GXM mAbs of this PS (GXM10-Ac_3) consists of 1.66 RU, a five-Man backbone, three of which are *O*-acetylated, with the central Man attached to a GlcA and the four lateral ones to Xyloses. Using NMR, we obtained localized distance and torsion information on GXM10-Ac_3, and then ran a 2 μs MD simulation of GXM10-Ac_3, which yielded an averaged structure that agreed with the NMR NOE and *J*-coupling data. Overall, these studies provided a representative 3D model of GXM10-Ac_3.

Next, we ran MD simulations on a 12 RU polymer of GXM (72 residues) which we correlated with the experimentally derived model of GXM10-Ac_3. We showed that the PS maintains a structural identity close to the previously derived GXM10-Ac_3 shape. We did this by monitoring each of the 11 GXM10-Ac_3 overlapping segments contained in the 12 RU PS and comparing with the experimentally derived model of GXM10-Ac_3 by RMSD throughout the MD duration, until the PS reached a steady state. The PS forms a flexible helical pattern, but with wide fluctuations in overall shape. The predicted pitch of the helical pattern, however, is longer than GXM10-Ac_3 segments (2.2 RU versus 1.66 RU). Analysis of the derived model indicates the pairing of *O*-acetyl groups alternating sides along the PS as key for Ab recognition.

In summary, we developed a method from which experimentally derived models of oligosaccharides can be utilized to correlate with MD trajectories of PS.

(P-148) Filling the Glycan Gap: how glycans modulate GABA Receptor function

Vidhi Sehrawat[1,2], Cyril Hanus[3,4], Mateusz Sikora[1,2]

[1]Malopolska Centre of Biotechnology, Jagiellonian University, 31-007 Kraków, Poland, [2]Department of Theoretical Biophysics, Max Planck Institute for Biophysics, 60438 Frankfurt, Germany [3]Institute of Psychiatry and Neurosciences of Paris, Inserm UMR1266, Université Paris-Cité, 75014 Paris, France, [4]GHU Psychiatrie et Neurosciences de Paris, 75014 Paris, France,

Inhibitory signaling in the brain primarily relies on glycosylated GABA receptors (GABARs), ion channels located at postsynaptic neuronal membrane. Glycans cover not only the outer surface of GABA, but also their inner vestibule, hinting at their role in ion conduction, receptor clustering, or binding of ligands (e.g., benzodiazepines). Although glycosylation seems indispensable for receptor function and its pattern varies depending on their neuronal location, the role of glycans in GABA mechanics remains poorly characterized. To address this gap, we simulated GABA receptors mimicking native neuronal environment using molecular dynamics (MD) simulations and investigated how glycans affected receptor dynamics and their interaction with ligands. Our preliminary findings expose glycan-specific influence on drug binding sites, ion entry into the vestibule, and surface shielding by glycans. These insights enhance our understanding of neuronal receptors and could aid in the creation of targeted, glycan-aware therapies.

Session 7: Gaining Insights into Glycoenzymes and Glycoconjugates with: High-throughput Analytical Approaches

(Key7-001) Making use of informative glycan-specific ions in glycopeptide MS/MS spectra

Kathryn Kothlow[1], Emmajay Sutherland[1], Tim S. Veth[1], Jacob H. Russell[1], Kayla A. Markuson[1], Anna G. Duboff[1], Ruby Zhang[1], Nicholas M. Riley[1]

[1]Department of Chemistry, University of Washington, Seattle, WA, USA,

B-type glycan fragments, often referred to as oxonium ions, are a result of fragmentation of mono- or polysaccharides. These glycan-specific fragments can inform a number of features about glycans that modify glycopeptides, including glycan class, composition, and structure. Oxonium ions are commonly used for several purposes in glycoproteomics and beyond, including but not limited to: determining the presence of glycopeptides in a dataset, examining gradient suitability for glycopeptide separations, classifying glycans based on known fragmentation ratios, evaluating glycopeptide identifications, and potentially informing glycan structure. Most studies rely on manual inspection to understand the oxonium ion content in their data, or they must use glycopeptide identifications to inform their inspection of oxonium ions. Here we develop an automated tool called GlyCounter to enable simple and accessible access to more than 40 oxonium ion features to explore glycopeptide content in raw mass spectra. We provide information about oxonium ion abundance, ratios to each other, and distribution across the chromatogram. We first show how GlyCounter can be used to improve glycopeptide separations through gradient optimization. We also use GlyCounter as a tool to quickly evaluate enrichment efficiencies. Finally, we demonstrate how to use GlyCounter to refine glycopeptide search strategies. Using the correct glycan database is key to high fidelity glycopeptide identification, but most studies do not generate sample-specific glycan databases. Instead, generic, literature-based glycan libraries are used. Searching with a glycan database that includes many glycan compositions that do not exist in the sample increases search times and false positive rates, thus reducing sensitivity. Conversely, omitting key glycan features (e.g., acetylation or specific glycan monomers) causes potentially identifiable and biologically relevant glycans to go unidentified. GlyCounter prevents these challenges by reporting glycan content of samples prior to glycopeptide searches. In all, we present GlyCounter as a new tool to evaluate the glycan content of glycoproteomic data that is decoupled from glycopeptide identification, ultimately enabling method refinement in sample preparation, data acquisition, and post-acquisition identification.

(Key7-002) New Analytical Technologies & Tools for Exploiting the Human Glycoproteome for Personalized Medicine

Melinda Wojtkiewicz[1], Sabarinath Peruvemba Subramanian[1], Roneldine Mesidor[1], Linda Berg Luecke[1], Jack Littrell[1], Rebekah L. Gundry[1]

[1]CardiOmics Program, Center for Heart and Vascular Research, and Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, 68198, USA,

Cell surface glycoproteins and glycans play critical roles in a range of physiological functions and disease processes, are valuable drug targets, and may be exploited as biomarkers for precision medicine. Despite their biological relevance and utility, glycoproteins and glycans are often understudied largely due to technical challenges. This presentation will describe CellSurfer 2.0 and glyPAQ, two complementary analytical platforms that enable rapid identification and quantification of cell surface glycoproteins and glycans, respectively, from small sample sizes. The application of these new methodologies to address outstanding questions in cardiac physiology and disease, with an emphasis on precision medicine, will be described. New developments in software, robotics, and mass spectrometry hardware that are especially critical for these workflows will be highlighted.

(Key7-003) LC-MS/MS-based structural glycoproteomics

Joshua C.L. Maliepaard, Weiwei Wang, Karli R. Reiding

[1]*Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University,* [2]*Netherlands Proteomics Center, Utrecht, The Netherlands*

Isomerism is an important issue in the field of glycosylation analysis. Glycans ubiquitously cover our cells and proteins, often being the first barrier of inter-cellular communication and pathogen-host interactions, as well as being the most-accessible biomarker reservoir. Glycosylation has long been studied by mass spectrometry (MS). However, while glycans have a multitude of isomeric properties, including fucose position, sialic acid linkage and antennary branching structures, these are not readily determinable by MS. For released glycans, this can be partially overcome by orthogonal means, e.g., ion mobility, liquid chromatography (LC) using porous graphitic carbon, or chemical derivatization, but for glycopeptides these techniques are not directly compatible. At the same time, only at the glycopeptide level can glycans be positioned at given glycoproteins and glycosylation sites.

To enable structural glycoproteomics, we present a combination of MS/MS-based structural glycopeptide characterization and high-resolution nano-HILIC-LC. As we demonstrate on structurally-defined glycopeptide standards, fragment ion ratios follow a distinct path through collision-energy-space depending on the structural characteristics. While this itself can already be used to quantify isomer ratios from a mixture, it also allows structural characterization of glycopeptide isomers that show chromatographic separation, for which we have optimized HILIC-LC. By applying the resulting LC-MS/MS methodology on complex samples, we reveal, for instance, distinct branch asymmetries for both recombinant and plasma-derived IgG subclasses.

(Key7-004) High Throughput Approaches to N-Linked Glycomic and Glycoproteomic Studies in Clinically Derived Human Tissues, Cells, and Fluids

Danielle Scott[1], Denys Rujchanarong[1], Stephen Zambrzycki[1], Harrison Taylor[1], Lyndsay Young[1], Jake Dressman[1], Anand Mehta[1], Richard Drake[1], Peggi Angel[1]

[1]*Medical University of South Carolina, Proteomics Center, Bruker Center for Clinical Glycomics,*

N-glycosylation plays a significant role in immune cell recruitment, alters disease progression, outcome, and response to therapy. Here, we will present an overview of recent advances in automated, rapid, and simplified workflows reporting N-glycomic and glycoproteomic expression in tissues, cells, and biofluids. These workflows have been purposefully developed to integrate closely with sample types from pathology labs used in diagnosis, tissue banks, and basic science laboratories. Case studies covering breast cancer risk, triple negative breast cancer outcomes, and applications in basic science application will be used to highlight the capabilities of these workflows. N-glycomics in molecular histology of the normal breast tissue microenvironment will be discussed relevant to incorporating clinical monitoring for cancer, socioeconomic stressors, genetic ancestry and life factors as risk factors for cancer. Prognostic potential will be highlighted by novel multiplexed N-glycomic studies on outcomes in triple negative breast cancer. New high throughput applications involving rapid scanning of N-glycans and glycoproteomics from drug treated or genetically altered cell types down to single cells will be presented. The developed workflows allow comprehensive studies on glyco-expression from bedside to bench and back to bedside, suitable for prognosis, diagnosis, biomarker discovery, and therapeutic efficacy.

(PT-018) Characterization of CD107a, CD107b, and CD68 glycosylation and its implication in the tumor microenvironment

Valentina Rangel-Angarita[1], Lea M. Kim[1], Ryan J. Chen[1], Nara Chung[1], Taryn M. Lucas[1], Keira E. Mahoney[1], Stacy A. Malaker[1]

[1]*Department of Chemistry, Yale University, New Haven, CT 06520, USA,*

The tumor microenvironment (TME) contains a variety of immune cells, with tumor associated macrophages (TAMs) being the most abundant. The surface of macrophages is composed of members of the lysosome-associated membrane protein (LAMP) family, specifically CD107a (LAMP-1), CD107b (LAMP-2), and CD68 (LAMP-4). Increasing evidence points to the significance of LAMPs in cancer, as they have been shown to play various roles in tumor progression. In particular, their predicted N- and O-glycan structures are thought to play a role in various cellular functions, including immune receptor binding, cellular adhesion promotion, tumor invasion, and metastasis. Despite this, structural characterization of their glycans has been limited, thus leaving a critical blind spot in understanding the functional and structural roles of LAMP glycans in TAMs and in cancer.

To address this gap in knowledge, we performed comprehensive glycoproteomic mapping of these proteins. Through multi-enzyme digestion and LC-MS/MS analysis, we uncovered a total of 17, 16 and 9 N-glycans in CD107a, b, and CD68, respectively. We noted a diverse collection of N-glycan structures, where some sites carried exclusively high mannose glycans, while others bore complex structures with LacNAc repeats. As for O-glycans, 17, 13, and 55 structures were site-localized in CD107a, b, and CD68. Importantly, CD68 carried a diverse repertoire of O-glycans, including heavy sialylation. Through this work, we identified and validated over 1500 glycopeptides modified by over 50 different glycan structures.

As CD68 is a mucin, we sought to further characterize its glycosylation changes and their potential implications in the TME. Using THP-1 cells, we studied glycosylation changes in different macrophage differentiation states. To do so, we enriched mucins from cell lysates using StcE-E447D, digested them, and analyzed using LC-MS/MS. We detected CD68 as one of the most abundant mucins, achieving near 100% coverage of its mucin domain. We identified 50 different glycoforms, including glycosylation changes between macrophage states. To investigate the role of CD68 glycosylation in the context of glycan-binding proteins, we conjugated CD68 onto solid support and incubated with HeLa lysate and cultured media. Proteins were eluted, digested, and analyzed via LC-MS/MS. We identified galectin-3 as significantly enriched through this process, which was previously unknown to interact with CD68. Future experiments are devoted to unraveling the glycoepitopes bound by galectin-3, as well as studying the interaction in more biologically relevant samples (e.g., PBMCs).

Overall, our work represents a comprehensive characterization of glycosylation modifications on the LAMP family of proteins, with a particular focus on the biological role of mucin CD68 in TAMs and inflammation. This research lays important groundwork for targeting these proteins in cancer, immune exhaustion, and inflammation treatments.

(PT-020) Single Cell Glycomic Analysis of Slide Captured Immune Cells

Lyndsay E.A. Young[1,2], James W. Dressman[1], M. Furkan Bayram[1], Richard R. Drake[1,2], Anand S. Mehta[1,2]

[1]*Department of Pharmacology and Immunology, 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*

Peripheral Blood Mononuclear Cells (PBMCs), which include lymphocytes, monocytes, and dendritic cells, play a pivotal role in the immune response and are readily accessible through standard blood collection. PBMCs can reflect the systemic metabolic environment, serve as a proxy to understand the broader metabolic disturbances occurring in various metabolic diseases such as cancer, and bridge the gap between basic scientific discoveries and clinical applications. We have recently developed a high-throughput antibody capture cell array-based slide using antibodies to CD4, CD8, CD19, and CD14 to determine the N-glycosylation patterns of each immune cell type in human PBMC isolates. Using PDMS stamps with defined special coordinates, a single cell capture version of the antibody slide array was created. Software programs to select areas that contain a single captured cell and record its slide location were also developed. Captured single cells were incubated with sprayed PNGase F to release N-glycans, followed by detection using a timsTOF fleX MALDI-QTOF mass spectrometer (Bruker, Billerica MA). Several thousand single cell N-glycome profiles of 40 structural compositions can be determined in 10 min or less. Differential abundances of detected N-glycans can readily distinguish the different immune cell types. The array slide format is inherently flexible for use of other capture antibodies, as well as lectins. It also allows sequential multimodal analysis workflows of the same captured cells, including detection of 250+ lipid species, 40+ N-glycans, 20+ glycogen derived glucose polymers, and 40+ tryptic peptides. Differentiating each from each immune cell subtype that cleanly differentiate CD4 and CD8 T-cells, and CD19 B-cells. This multimodal interrogation enhances our knowledge of the functional N-glycosylation patterns in the active immune system, and offers potential biomarkers for identifying patients who might benefit from specific immunotherapeutic interventions.

(PT-022) Specific Modifications of N-glycans are Restricted to Synapses of Specific Neurons

Maxence Noel[1], Dongli Lu[1], Ea Tulin[1], Lukas Muerner[2], Suttipong Suttapitugsakul[1], Lauren Pepi[1], Stefan von Gunten[2], Robert G. Mealer[3], Richard D. Cummings[1,*]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA,* [2]*Institute of Pharmacology, University of Bern, Inselspital, INO-F, Bern 3010, Switzerland* [3]*Department of Psychiatry, Oregon Health & Science University, Portland, OR 97239,*

The glycome of the brain is amazingly complex and its characterization represents a modern challenge that offers great promises to better understand the functions of glycans. Sulfation of glycans is a modification that can occur in all types of glycans and has important roles in brain function. Our recent study focusing on mapping the brain N-glycome showed that specific sulfated N-glycans were present in unique regions of the brain. However, common glycan-binding proteins that are able to recognize this modification have a limited specificity in their recognition, highlighting the need of developing new reagents to better define the brain glycome. In regard to the specific modification of terminal galactose residues with sulfate (3-O-sulfated galactose) we used both mass spectrometry analysis along with an antibody produced by immunization of sea lampreys and found to be specific to recognizing 3-O-sulfated galactose. This antibody allowed us to precisely localize these structures within brain slices using immunofluorescence and perform enrichment techniques to identify their protein carriers through proteomics. In this study we identify: 1) the specific distribution of sulfated N-glycans in the cerebellum, hippocampus and a layer of the cortex along with a correlation with the molecular identity of the Purkinje cells that highly express this determinant; 2) the carrier proteins modified by 3-O-sulfated galactose, which were found to associated with cell adhesion, pre- and post-synaptic compartment and glutamate receptors; and 3) by using CRISPR-Cas9 technology, we identified *Gal3ST3* as the gene involved in the synthesis of this epitope and therefore identified the subtypes of neurons expressing this enzyme by transcriptomics analysis. This study is one of the first to deeply characterize the 3-O-sulfation of galactose and unravel a strict distribution in the brain, which will help in characterizing the unexpected restricted expression of glycans in the brain and further our understanding of this modification to neuronal functions in the brain.

(PT-024) Discovery of high efficiency oligosaccharyltransferase mutants for conjugate vaccine synthesis using a high-throughput cell-free screening platform

Zachary M. Shaver[1,2,3], Derek A. Wong[1,2,3,4], Michael C. Jewett[1,2,3,4]

[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,* [4]*Department of Bioengineering, Stanford University, Palo Alto, CA, USA,*

Glycoconjugate vaccines, composed of bacterial capsular polysaccharides or O-antigen glycans conjugated to immunogenic carrier proteins, are an effective strategy to promote immunity and prevent bacterial infections. The conventional method to synthesize conjugate vaccines uses chemical techniques to non-specifically attach bacterial glycans to a purified carrier protein. While functional, this process is expensive, requires growing pathogenic bacteria, and modifies protective immune epitopes on the carrier. Recently, enzymatic conjugation methods have been developed in both cell-based and cell-free systems to conjugate glycan antigens to carrier proteins using an oligosaccharyltransferase, such as PglB from *Campylobacter jejuni*. This enzymatic method addresses the limitations of chemical conjugation and enables the synthesis of a homogeneous and more highly immunogenic vaccine product. Unfortunately, enzymatic conjugation is currently limited by low glycosylation efficiency – the proportion of aglycosylated carrier protein that becomes glycosylated in an enzymatic reaction. Improving glycosylation efficiency is therefore essential to increase vaccine yield and produce more vaccine doses per reaction. In this work, we evaluated the effect of mutating the oligosaccharyltransferase PglB on the enzyme's glycosylation efficiency. We designed a library in which 15 amino acids at chosen sites within PglB were mutated to all 19 other amino acids, creating a library of 285 single-mutants. We used cell-free protein synthesis to express each mutant and added the mutants to *in vitro* enzymatic reactions. AlphaLISA, an in-solution bead-based ELISA assay, was then performed to detect carrier glycosylation and evaluate each mutant's glycosylation efficiency in high-throughput. In total, 7 mutants demonstrated higher glycosylation efficiencies than wildtype PglB, and AlphaLISA results for both high- and low-signal mutants were confirmed by western blot. Furthermore, 7 unique amino acids that were chosen for mutation generated at least one mutant with signal > 5× above background. This work reveals numerous novel mutations within an oligosaccharyltransferase that improve its glycosylation efficiency. Future research will expand this mutagenesis screening towards an array of pathogen glycans to improve enzymatic conjugation technology and synthesize novel conjugate vaccines.

(P-149) Establishing a Glycogene Knockout Library in Breast Cancer Cells

Ramiz Demir[1], Lara K. Mahal[1,*]

[1]*Department of Chemistry, University of Alberta, Edmonton, Alberta,*

*Corresponding Author

Glycosylation is a critical part of cellular homeostasis, and dysregulation of the biosynthetic enzymes modulating glycosylation leads to various cellular abnormalities, including cancer. To date, the connection between biosynthetic glycosylation enzymes, glycan structures and the molecular mechanisms of disease are still unclear. To shed light on these connections, we aimed to create a CRISPR glycogene knockout cell line library of the classic breast cancer cell line, MCF7. To create this cell library, we leveraged the guide RNA (gRNA) library of Neelamegham et al (Addgene #140961)[ref] which targets 347 genes that have function in the glycosylation process with 10 guides per gene. The cell line library was created in two main steps; integration of 1) the Cas9 gene and 2) gRNAs into the genome of the cell line. Cell sorting was performed to obtain cells expressing Cas9 and gRNAs following sequential lentiviral transductions. We validated the cell set by RNA sequencing. This cell line library is a powerful tool to probe the role of glycosylation in cancer biology and the role of glycan enzymes in establishing glycan structures. Future work utilizing this library will open up new horizons in glycoscience.

(P-150) SAGRs (Smart Anti-Glycan Reagents) to Brain Glycans: Novel Glycan-specific Antibodies Target Brain Neurons, Endothelial Cells, and Nodes of Ranvier

Ea Kristine Clarisse Tulin[1], Maxence Noel[1], Lukas Muerner[2], Mary H. Florido[3], Louie Cielen[4], Lauren Pepi[1], Vivianne Otto[4], Richard D. Cummings[1]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, National Center for Functional Glycomics, Harvard Medical School, Boston, MA,* [2]*Institute of Pharmacology, University of Bern, Bern, Switzerland* [3]*Harvard Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts, USA,* [4]*Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland,*

Glycosylation plays a role in neural cell interactions and modulates several neurological disorders. Recent advances in analyzing glycosylation have allowed us to characterize the brain glycan profile in various contexts during development and disease. However, the role that many glycans play in the central nervous system (CNS) remains elusive, and tools to carefully map specific expression of glycans are limited. Glycan-specific monoclonal antibodies are vital to visualizing the distinct localization and targeting of glycans in the CNS. Previously, we reported the production of single monoclonal antibody reagents (SAGRs - Smart Anti-Glycan Reagents) discovered from immunized sea lamprey that have different glycan specificities - OmcFL3-02 broadly targeting H type antigens, Tn4-31L specific for H type II antigens, and PBMC3-02 specific for terminal Neu5Aca2-6. Using these SAGRs as recombinant antibodies produced as Ig chimeras, we map the localization of terminal fucose and sialic acid in the mouse brain and reveal *N*- and *O*-glycan targets using glycosidase treatments. Furthermore, we demonstrate that H type *O*-glycans are enriched in brain endothelial cells, H type II *N*-glycans in neurons that cluster the accessory olfactory bulb, and Neu5Aca2-6 terminal glycans in the Nodes of Ranvier. Myelination, olfaction, and the blood-brain barrier are three critical areas of CNS function, and the roles of glycans containing terminal fucose and sialic acid in these processes remain poorly understood. Our findings highlight the utility of SAGRs for analyzing brain glycosylation, providing a paradigm for investigating neuro-glycobiology in brain development and human pathologies.

(P-151) Development of Functionalities for Glycopeptide MS Data Analysis

Masaaki Matsubara[1], Issaku Yamada[1]

[1]*The Noguchi Institute,*

At our institute, the development of comprehensive methods for analyzing O-linked glycans is significantly hampered by the extensive manual analysis required for mass spectrometry (MS) data. The manual sorting of peaks and spectral data from enzymatically digested glycopeptides proves to be particularly resource-intensive. While there is a pressing need for software to streamline these tasks, current offerings for complex glycoproteins fall short. Some proteomics analysis software, when customized, can be adapted for glycan analysis; however, they often suffer from high false positive rates. Additionally, recent developments in glycoproteomics software are typically limited to N-linked glycans and offer limited functionalities, making them unsuitable for our specific needs. To address these challenges, we have embarked on developing software that aids the

analysis of glycopeptide MS data. An investigation into our workflows revealed that the most labor-intensive process involves detecting peaks from glycan-derived fragments in extensive MS/MS spectra. To facilitate this, we are developing a feature that allows for the detection of MS/MS spectra containing peaks with m/z values of glycan-derived fragment ions. A key component of this feature is a mass calculation function that simplifies the input of m/z values for glycan and peptide fragment ions. Users can input monosaccharide compositions and amino acid sequences in a designated format to automatically calculate their masses and, with specified ions and neutral losses, their m/z values. These calculated names and m/z values can be registered as conditions for MS/MS spectra detection. The results obtained through this feature can be viewed in formats such as tables or spectra. Currently, these functionalities are implemented in the MS data viewer Mass++ v4. Moving forward, we plan to enhance accessibility for the glycan research community by ensuring compatibility with existing glycan MS data analysis software like GlycoWorkbench.

(P-152) A General Method for High-Throughput Multiplex Glycan Bead Array and Neoglycoprotein Library to Profile Glycan-Binding Proteins and Antibodies

Tianwei Jia[1], Richard D. Cummings[*,1]

[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, National Center for Functional Glycomics, CLS 11087-3 Blackfan Circle, Boston, Massachusetts 02115, United States,*

Elucidating the specifics of diverse glycan-binding proteins (GBPs) is crucial for understanding glycan-mediated biological recognition events and pathways, such as cell-cell communication, organismal development, and immune responses. Here, we present a general method for preparing a high-throughput Luminex multiplex glycan bead array and a neoglycoprotein library to investigate GBP-glycan interactions. Utilizing a novel multifunctional linker, 3-(methoxyamino)propylamine addition a biorthogonal-functional tetrazine (MTZ), we derivatized a library of glycans while preserving their structural integrity. The glycans were rapidly conjugated to avidin Luminex beads through a Biotin-PEG11-TCO spacer using Biotin/Avidin and TCO/Tetrazine interactions. This bead array was successfully interrogated by various lectins, antibodies, and pooled intravenous immunoglobulin (IVIG). We profiled anti-glycan IgG, IgM, and IgA antibodies in 13 human serum samples, with each sample diluted serially from 1:100 to 1:6400. Our findings revealed that each individual's repertoire of anti-glycan antibodies is relatively unique. Additionally, the neoglycoprotein library was prepared using a similar strategy, with different glycans conjugated to bovine serum albumin (BSA), resulting in a consistent average number of glycans ranging from 18.5 to 23.9. The neoglycoprotein microarray was evaluated with lectins and IVIG, yielding results comparable to the bead array. Neoglycoproteins BSA-LNT and BSA-LNnT were employed to develop an ELISA assay for investigating ST6Gal1 sialyltransferase activity. The ELISA assay demonstrated a two-fold higher binding affinity of ST6Gal1 for BSA-LNnT compared to BSA-LNT, suggesting that ST6Gal1 has limited activity toward LNT but high activity toward LNnT, thus providing a structural basis for its substrate preference.

(P-153) Characterization of the N-Glycosylation of Native and Recombinant Human Lactoferrin

Xiaoning Lu[1,*], Chad Cummings[1], Udodili A. Osuala[1], Pamela B. Besada-Lombana[1], Ross D. Peterson[1], Anthony J. Clark[1]

[1]*Helaina Inc., 345 Park Avenue South, 5th Floor, New York, NY 10010, USA,*

*Corresponding author

Human lactoferrin (LF) is an iron-binding glycoprotein found in high concentrations in human milk and to a lesser degree in tears, saliva, intestinal secretions, and neutrophils. Human milk LF is a bioactive protein that nourishes newborns and supports optimal growth and development. Lactoferrin also supports immune health at every life stage. There is a desire to use recombinant genetic technology to develop sustainable methods for the industrial scale production of human LF. Helaina recombinant human LF (effera^{TM}) is the first of its kind that was produced at an industrial scale.

We have employed two independent approaches to gain a full understanding of the N-glycosylation of effera and native human milk LF, both employing high-resolution mass spectrometry. In the first approach, LF was digested by trypsin and the resulting peptides including the glycopeptides were identified. In the second approach, the N-glycans were released from LF by PNGaseF and then detected and identified by mass spectrometry as well as fluorescence detection. N-linked glycans were detected at three known glycosylation sites for both effera and native human milk LF, namely, Asparagines-156, 497, and -642. The results showed the N-glycans of effera were predominantly oligomannose structures having five to nine mannoses. The analysis also identified that the N-glycans of effera are oligomannose structures having five to 14 mannoses (M5-M9), with the smaller N-glycans M5-M9 taking account for about 60% of the total N-glycans. Additionally, M5 and M6 were detected in the native human milk LF samples although complex N-glycans are the predominant ascribed glycoforms. Lastly, we were also able to

identify the glycoforms of effera and native human LF by the measurements of the intact protein masses by the high-resolution mass spectrometry.

(P-154) Structural Analysis of Glycans in Complex Biological Samples Utilizing a Library of Glycan Standards

Lauren E. Pepi[1,2], May Wang[1], Christopher Ashwood[1,2,3], Lukas Muerner[1,4], 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,* [4]*University of Bern, Bern, Switzerland,*

Protein glycosylation is a common but variable modification found in all living organisms. The biosynthetic pathway for glycans is a non-template driven enzymatic process, resulting in structural variation and complexity. The use of liquid chromatography coupled to mass spectrometry (LC-MS) allows for the separation of glycans; however, without standards, it is extremely challenging to determine exact structure. Our lab has acquired over 200 glycan standards from different vendors with defined structures that were utilized to develop a targeted MS/MS assay using diagnostic fragment ions. This method distinguishes sialic acid linkage, *N*-glycan arm extensions, bisecting N-acetylglucosamine, and fucose position. Herein we have applied this method to two separate cell lines and three different gene knockouts which have differing glycan profiles. Glycan standards were analyzed individually and as mixtures to characterize their fragmentation patterns and elution order. Collision energies were optimized for each standard, and this information was used to develop targeted methods for *N*-glycans, *O*-glycans and free oligosaccharides. HEK293 and K562 cells with varying gene knockouts were processed for the glycolipids, *N*-glycans and *O*-glycans.

MS analysis was performed using a Thermo Fisher TSQ Altis Plus Mass Spectrometer coupled to a Vanquish liquid chromatography system. A Hypercarb column was used to separate glycan samples over an 80-minute gradient for *N*-glycans, and a 40-minute gradient for free oligosaccharides and O-glycans. Wild type (WT) HEK293 and K562 cells, as well as cells engineered to lack specific glycogenes, including *MGAT1*, *UGCG* and *CMAS* knockouts (KO) for both cell lines, were analyzed for their *N*- and *O*-glycans and released oligosaccharides from glycolipids. Over 60 *N*-glycans, 15 *O*-glycans and 9 free oligosaccharides were identified across all samples.

(P-155) Machine Learning-driven Surface Enhanced Raman Spectroscopy Sensor for Glycosylation Analysis

Qiang Hu[1], Aman Yadav[1], Assame Arnob[1], Hung-Jen Wu[1]

[1]*Chemical Engineering Department, Texas A&M University,*

Monitoring glycosylation patterns is crucial for understanding biomolecular mechanisms, predicting biological consequences, and controlling the quality of therapeutic proteins. Because glycosylation is a post-translational modification, frequent monitoring of glycosylation levels at different growth conditions is necessary. Staining glycan samples with glycan-binding molecules, such as lectin and chemical probes, are a popular analysis. Nonetheless, given the complex nature of glycans, designing numerous molecules with desired properties to recognize different glycan structures is unrealistic. To overcome the challenges, a different detection strategy is developed. This new sensing platform combines three major components, including a Boronic Acid (BA) array, Surface-Enhanced Raman Spectroscopy (SERS), and an advanced Machine Learning (ML) model. BAs that could react with the hydroxyl groups on glycan molecules are used to capture glycans from complex biological samples. Such a reaction is not highly specific to a particular glycan structure; therefore, the yes/no confirmative response (e.g., fluorescence) in the staining assay is not sufficient for glycan analysis. In order to recognize the structural differences, this new sensor monitors molecular fingerprint spectra. Different types of glycans can induce different vibrational spectra after reacting with BAs; therefore, glycan structures can be identified by analyzing their Raman spectra. SERS technique that offers strong Raman signal enhancement is used to improve the detection limit. The availability of low-cost Raman spectrometer and SERS substrates will make this sensing platform immediately available for a wide range of users. Moreover, complex Raman spectra will be interpreted using the advanced ML program. The ML model can predict the glycan structures and compositions solely based on the experimental spectra. End users who do not have knowledge of bioinformatics can conduct data analysis using sophisticated ML software. Here, we established a BA receptor array to improve the detection accuracy. This sensing platform could directly analyze the biological samples, including the whole milk and intact glycoproteins (fetuin and asialofetuin), without tedious glycan release and purification steps. The results demonstrate the platform's ability to classify oligosaccharides with remarkable classification accuracy, despite the presence of other non-glycan constituents in the background. This sensor could also directly quantify

glycosylation levels of glycoproteins (e.g. sialylation levels in fetuin/asialofetuin mixture) without glycan release procedures. Moreover, by selecting appropriate BA receptors, the sensor exhibits an excellent performance of differentiating glycosidic linkages (e.g. α2,3 and α2,6 linkages of sialic acids). This low-cost, rapid, and highly accessible sensor will provide the scientific community with an invaluable tool for routine glycan screening in standard laboratories.

The prototype sensor will be validated using in vivo Drosophila models, and the inter-comparison test and cross validation will be conducted to demonstrate sensor robustness and adaptability. This new sensing platform is highly transformative, because: (1) The detection principle is universal, so the sensor can study various types of glycosylated molecules (e.g. glycoproteins and glycolipids) without labor-intensive sample preparation. (2) The cost of the detection is low. (3) The simple protocol allows end users to frequently conduct the assay in their own laboratories without expensive equipment. (4) Transformer-based ML software will assist end users who do not have bioinformatic knowledge in completing complex data analysis.

(P-156) Characterization of Bone Morphogenic Protein Receptor 2 *N*-Glycosylation Reveals High Heterogeneity at Site Involved in Ligand Binding

Valeria Nazaire[1], Carlos H. Pavan[1], Kevin Brown Chandler[1]

[1]*Translational Glycobiology Institute, Department of Cellular and Molecular Medicine, Florida International University Herbert Wertheim College of Medicine, Miami, FL 33199,*

Bone morphogenic protein receptor 2 (BMPR2) is an *N*-glycosylated serine/threonine kinase receptor with three *N*-glycosylation sequons within its extracellular domain. In the lungs, BMPR2 influences endothelial cell proliferation, cell death, and angiogenesis. Recently, it has been reported that BMPR2 glycosylation influences ligand binding and activation, with potential implications for pulmonary health. Loss of function mutations in the gene encoding BMPR2, including mutations that abolish *N*-glycosylation sites, are associated with heritable pulmonary arterial hypertension (PAH), an irreversible condition characterized by increased blood pressure in the pulmonary arteries potentially leading to heart failure and death. However, BMPR2 glycosylation has not been fully characterized, and the role of glycosylation in BMPR2 stability and signaling is not well understood. Given the potential importance of the BMPR2 *N*-glycans in receptor activity, we sought to characterize the three putative *N*-glycosylation sites. To accomplish this, nano-flow liquid chromatography tandem mass spectrometry (nLC-MS/MS) was utilized to analyze BMPR2 proteolytic products. Byonic and pGlyco3 software were used to assign peptides and glycopeptides. Based on these analyses, *N*-glycopeptides occupying all three putative *N*-glycosylation sites on BMPR2 (N29, N84, and N100) were assigned with high confidence. High coverage of BMPR-2 was achieved, and site-specific *N*-glycan heterogeneity was documented at all three sites, including glycan compositions consistent with the presence of fucosylated and sialylated *N*-glycans. Extensive *N*-glycan heterogeneity was observed at site N110, involved in BMPR2 ligand binding. This characterization of BMPR2 *N*-glycosylation will serve as the foundation for future studies focused on understanding the role of BMPR-2 *N*-glycosylation in ligand binding.

(P-157) *N*-GlyFind™ – A High-Specificity Affinity Reagent for Detection and Enrichment of *N*-Glycosylated Proteins

Sheng-Cheng Wu[1], Christian Gerner-Smidt[1], Lu Meng[1], George N. Bendzunas[1], Sylvain Lehoux[1], Robert J. Woods[2], Loretta Yang[3]

[1]*Lectenz Bio, Athens, GA,* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA* [3]*Lectenz Bio, San Diego, CA,*

Asparagine-linked glycans (*N*-glycans) play crucial roles in nearly every aspect of biological processes, and their distinct properties make them appealing as disease biomarker and therapeutics targets. However, due to their highly branched and variably linked nature, *N*-glycans remain a challenge to detect, purify, and analyze structurally. Despite current advances in analytical techniques and instrumentation, there is still a great need for high-affinity reagents with well-defined epitope specificity that can be used to interrogate and enrich biological samples. Lectenz Bio has been engineering glycan-processing enzymes and glycan-binding proteins into high-affinity glycan-binding reagents with tunable specificities. Here, we report the development of *N*-GlyFind™, an *N*-glycan detection reagent engineered via directed evolution from a mouse F-box only protein 2 (FBXO2 or Fbs1). Our approach harnesses molecular dynamics (MD) simulations to explore the dynamic nature of protein-glycan interactions, enabling the identification of specific amino acid residues for construction and screening of a combinatorial yeast display library. The resulting *N*-glycan core-specific candidates were further validated by a panel of assays such as glycan microarray, Western blot, Bio-Layer Interferometry, ELISA, and affinity chromatography. The lead candidate, called

N-GlyFind^{TM}, has been identified as a pan-specific *N*-glycan affinity reagent exhibiting high selectivity towards glycoproteins and peptides with various *N*-linked glycoforms.

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(P-158) Ultra-deep O-GlcNAc proteomics reveals widespread O-GlcNAcylation on tyrosine residues of proteins

Junfeng Ma[1,*], Chunyan Hou[1], Jingtao Deng[1]

[1]*Department of Oncology, Georgetown University Medical Center, Washington DC, USA,*

*Corresponding author

O-linked β-N-acetylglucosamine (O-GlcNAc) is a post-translational modification (i.e., O-GlcNAcylation) on serine/threonine residues of proteins. Distinct from the traditional glycosylation (i.e., N-glycosylation, O-glycosylation, and GPI-anchored glycosylation), O-GlcNAcylation is a unique intracellular monosaccharide modification without being further elongated into complex sugar structures. O-GlcNAcylation has been documented in all metazoans (including animals, insects and plants), some bacteria, fungi and virus. O-GlcNAcylation is catalyzed by a discrete set of enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT transfers the GlcNAc moiety from UDP-GlcNAc to specific serine/threonine residues of target proteins while OGA removes it. Protein O-GlcNAcylation plays important roles in almost all biochemical processes examined (including transcription, translation, cell cycle, metabolism and signaling). Aberrant O-GlcNAcylation is underlying the etiologies of a number of chronic diseases (including cancer, diabetes, and neurodegenerative disease).

To pinpoint site-specific functions of O-GlcNAcylation, tremendous efforts have been made to map O-GlcNAc sites for either individual proteins of interest or entire proteomes. However, deep and site-specific O-GlcNAc proteomics remains a challenge. Very recently, we constructed an ultra-deep O-GlcNAc proteomics workflow, in which multiple-enzymatic digestion was coupled with an efficient O-GlcNAc enrichment method, two mass spectrometric approaches (i.e., EThcD fragmentation and HCD-pd-EThcD fragmentation), and two data analysis tools (i.e., MaxQuant and Proteome Discover). This integrated approach yielded unprecedentedly deep coverage of the O-GlcNAc proteome for PANC-1 cells, with 2831 unambiguous O-GlcNAc sites identified with high confidence (which is by far the biggest dataset reported). In addition to confirming known sites and discovering many novel sites of Ser/Thr modification, O-GlcNAc modification was found on > 120 tyrosine (Tyr) residues on proteins. Moreover, OGT was found demonstrating catalytic capacity to perform O-GlcNAcylation onto Tyr residues. In addition, in vitro assays suggest that OGA can remove the O-GlcNAc moiety from Tyr. Collectively our data show that 1) Tyr is a new target for O-GlcNAcylation and Tyr O-GlcNAcylation is a novel type of glycosylation; 2) Tyr O-GlcNAcylation is an enzymatic reaction potentially mediated by OGT and OGA, which may have important regulatory roles.

(P-159) Automating the Design of Glycomimetic Agents

Yao Xiao[1], Sawsan Mahmoud[1], Alex H. Lee[1], Robert J. Woods[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA,*

Introduction: Specific interactions between carbohydrates and proteins underlie the initiation or progression of many diseases. Carbohydrate-binding proteins (human, bacterial or viral lectins and adhesins) and carbohydrate-processing enzymes (glycosyltransferases and glycosidases) are therefore important targets for therapeutic intervention, however the creation of drug-like molecules that can competitively inhibit carbohydrate-binding sites is uniquely challenging. The optimization of a glycomimetic inhibitor involves the synthesis and screening of chemical analogs in an attempt to increase the inhibitory potential and biological activity. Despite the synthetic challenges, the benefit of employing the native carbohydrate as a scaffold is that it intrinsically confers the desired specificity. The fundamental challenge in the creation of a glycomimetic is that of divining which modifications will lead to enhanced affinity without compromising specificity.

Methodology: Here we present our work towards the development and validation of a computational strategy that leverages the benefits of computational modeling and structural biology. Specifically, we are creating an automated computational approach that uses carbohydrate-protein co-crystal (or NMR) structures as the basis for lead optimization by modifying the bound oligosaccharide *in situ*.

Results & Discussion: We have successfully constructed a library of 545 drug-like aliphatic and aromatic moieties, extracted from Sigma -Aldrich, ensuring that they are available for synthetic strategies. Each moiety may be conjugated at any available hydroxyl or amino position in a carbohydrate, which for a single monosaccharide with five available positions for derivatization gives rise to 545^5 (4.8×10^{13}) potential glycomimetic compounds. Here we will discuss the automated screening of this moiety library with

regard to several protein receptors, including influenza hemagglutinin. In the course of this work, we have noted weaknesses in current computational energy functions, including the lack of CH-π interactions, which we have recently corrected[1] in the popular docking software AutoDock VINA-Carb[2]. CONCLUSIONS: The successful completion of this project will facilitate the application of sophisticated modeling techniques by users who are either not experts in modeling, or not experts in carbohydrate chemistry.

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References

Xiao Y, Woods RJ. *J. Chem. Theory Comput.* 2023:**19**:5503–5515.
Nivedha AK, Thieker DF, Hu H, Woods RJ. *J. Chem. Theory Comput.* 2015:**12**:892–901.

(P-160) Collaboration, Service and Trainings at the Complex Carbohydrate Research Center

Varughese A. Mulamoottil[1], Christian Heiss[1], Artur Muszynski[1], Ian Black[1], Stephanie Archer-Hartmann[1], Jiri Vlach[1], Li Tan[1], Xu Yang[1], Bhoj Kumar[1], Nathan Murray[1], Lei Huang[1], Jie Lun Cheng[1], Sergei Shalygin[1], Mehrnoush Taherzadeh Ghahfarrokhi[1], Ambrish Kumar[1], Liyanage D.P. Fernando[1], Krishna Moorthy Manchuri[1], Hadi Tabani[1], Sonali Sunsunwal[1], Jose Alejandro Villalobos Jr[1], Marium Khaleque[1], Parastoo Azadi[1]

[1]*Analytical services, Complex Carbohydrate Research Center, University of Georgia, Athens, GA,*

The last three decades has seen a continuous rise in collaborations of the Complex Carbohydrate Research Center (CCRC) with universities, federal agencies, and industries around the globe, focusing on the detailed structural characterization of glycoconjugates. Our expertise spans a broad range of analytical methodologies essential for comprehensive glycobiology studies. We utilize cutting-edge instrumentation, including the 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, and multiple analytical and preparative separations options, and GC-MS. The CCRC's NMR facility offers outstanding resources for high-field NMR spectroscopy including the recently installed 1.1 GHz, along with the 900 MHz, 800 MHz, three 600 MHz and a 500 MHz spectrometers. 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 at the CCRC cover diverse areas such as glycomics, glycoproteomics, glycosaminoglycans (GAGs), Gag-derived products, polysaccharides, lipopolysaccharides (LPS), peptidoglycans (PGs), and glycolipids. Our team at CCRC excels in developing new analytical techniques or optimizing existing methodologies, to ensure that each method meets the unique requirements of our clients. Our approach not only advances the field but also provides customized support to overcome complex research challenges and achieve precise, reliable results.

The CCRC offers annual 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 augmented by faculty-led lectures that explore various facets of glycobiology.

(P-161) GlycoFASP: A Universal Method to Prepare Complex Mixtures for O-Glycoproteomic Analysis

Shane M. Finn[1], Keira E. Mahoney[1], Valentina Rangel-Angarita[1], Taryn M. Lucas[1], Stacy A. Malaker[1]

[1]*Department of Chemistry, Yale University, New Haven, CT, USA,*

Recently, a focus has been placed on developing more comprehensive enrichments for O-glycoproteomics. Many of these leverage O-glycoproteases, a class of endoproteases that cleave adjacent or near O-glycosylated residues. One such enrichment utilizes the catalytically inactive point mutant of mucinase StcE to isolate densely O-glycosylated proteins. Other strategies such as EXoO (extraction of O-linked glycopeptides) and O-GIG (O-Glycopeptide immobilization for O-glycosylated peptide enrichment) involve conjugating tryptic peptides onto solid support and releasing O-glycopeptides using OgpA (OpeRATOR, Genovis) and ImpA (O-glycoprotease, NEB). This strategy was recently expanded upon by using multiple O-glycoproteases in tandem in a technique the authors called MOTAI.

While these methods have been used to enhance analysis of the O-glycoproteome, they are not without shortcomings. StcE enrichment has a stronger affinity to mucins than to less-densely O-glycosylated proteins. Chemoenzymatic enrichments such as

EXoO, O-GIG, and MOTAI need multiple rounds of purification, which greatly reduces peptide recovery. Thus, an average of 40 mg of lysate is typically needed for a single glycoproteomic analysis. Further, these peptide conjugation approaches require around 4 days of processing time and require pre-fractionation, thus requiring another 2 days of instrument time. The many steps required for peptide-conjugation strategies make them prone to signal-splitting and side reactions, potentially rendering them ineffective for different sample types or causing a lack of reproducibility.

To address the pitfalls of current enrichments, we leveraged the advantages of a widely used proteomics technique called Filter Aided Sample Preparation (FASP). Here, detergent is depleted from samples using an ultracentrifugation filter, then digested on the same filter to generate highly pure peptides. Our approach, herein dubbed GlycoFASP, uses a one-pot sample preparation that requires no purification and can be fully processed and analyzed in 2 days. Samples are added to a molecular weight cut-off spin-filter membrane, digested with O-glycoproteases, and the flow-through is collected for MS analysis. In our initial experiments, we achieved nearly 1500 O-glycopeptide identifications from HEK cell culture media, over 250 from HeLa lysate, and over 400 from serum. Additionally, GlycoFASP requires only 1 mg of lysate – a marked 40-fold decrease – to obtain comparable O-glycopeptide ID counts to peptide-conjugation strategies. Our method is compatible with sequential digestions, like MOTAI, and can obtain near complete mucin-domain sequence coverage of a variety of proteins such as versican core protein, proteoglycan 4, and MUC1. Current efforts are involved in optimizing the filter membrane, sample amount, and enzyme ratio to maximize glycopeptide recovery.

(P-162) The role of miRNAs in modulating alpha-1,2-fucosyltransferase

Tigist Batu[1], Lara Mahal[1]

[1]*University of Alberta,*

Micro-RNAs (miRs) are 18–22 nucleotide base length non-coding RNAs that enable tight control of protein expression within the cell, strongly impacting low abundance proteins such as glycosyltransferases. Using our high-throughput fluorescence-based assay (miRFluR) we mapped the miRNA regulatory landscape of the antimetastatic gene FUT1, the enzyme responsible for adding fucose at alpha-1,2- position of galactose residue of N- and O-linked glycans. We find that FUT1 is predominantly upregulated by miRNA, contradicting the conventional role of miRNAs as downregulators of protein expression. Validation of our data in melanoma and other cancer cell lines confirmed the upregulation of FUT1 by both miRNA mimics and endogenous miRNAs. Mutational analysis on the strongest upregulatory miRNA sites i.e., miR200c-5p and miR-361-5p, identified direct non-canonical interactions underlying upregulation, deviating from conventional seed-pairing rules. Overall, this work sheds light on the regulation of alpha-1,2-fucosylation bidirectionally by small non-coding RNA.

(P-163) Glyco-PASEF®: Enhancing Glycoproteomic Analysis via Oxonium-Ion-Gating and Stepped-Energy CID on the timsTOF

Hongxia Bai[1], Kristina Marx[1], Florian Busch[1], Stephanie Kaspar-Schoenfeld[1], Ulrike Schweiger-Hufnagel[1], Gary Kruppa[1], Albert Heck[2], Klaus Lindpaintner[1]

[1]*Bruker-Daltonics, Life-Science Mass Spectrometry, Billerica/USA, Bremen/Germany, Fällanden/Switzerland,* [2]*University of Utrecht/ Netherlands*

Introduction: Accurate characterization and quantification of glycopeptides are essential for understanding their biological functions and identifying potential biomarkers for various diseases. However, the complexity of glycan structures and their diverse ionization and separation behaviors pose significant challenges. Integrating trapped ion mobility spectrometry (TIMS) with liquid chromatography-mass spectrometry (LC-MS) provides an enhanced separation dimension that is particularly advantageous for glycoconjugate analysis. Glycopeptides can be isolated more effectively from non-glycosylated peptides based on their unique ion mobility properties, reducing background noise and enhancing the analytical specificity. Also, different glycan and peptide backbone structures require specific fragmentation analysis energies for optimal characterization.
Methods: To explore the glycoproteomic landscape, we initially optimized the Parallel Accumulation Serial Fragmentation (PASEF) technique on the timsTOF Pro using unenriched tryptic peptides from purified glycoproteins and human plasma samples. Building on these promising results, we are currently evaluating the enhanced capabilities of the timsTOF Ultra 2. Key optimizations included implementing a glycan-specific polygon (GP) for targeted precursor selection and employing stepped collision energy (SCE) to improve fragmentation. We analyzed four strategies: standard PASEF, SCE-PASEF, GP-PASEF, and GP-SCE-PASEF. Spectra were generated by summing peak intensities across the m/z grid and processed into MGF-meta files containing precursor intensity, collisional cross-section (CCS), and monoisotopic mass. Data were analyzed with MSFragger, FragPipe, IonQuant, and Philosopher software to identify N-glycopeptides based on oxonium ions in MS/MS spectra.

Results: TIMS effectively separated N-glycopeptides from non-modified peptides, enhancing analytical depth for both purified glycoproteins and complex biological samples such as plasma. The glycan-specific polygon in PASEF mode, combined with SCE, significantly improved N-glycopeptide identification. The GP-SCE-PASEF-GP (glyco-PASEF®) method yielded a nearly tenfold increase in unique annotated glycopeptides compared to the original PASEF-only approach, identifying over 500 unique N-glycopeptides from unenriched plasma. The combination of glyco-polygon filtering with SCE resulted in a 20% and 51% increase in identified N-glycopeptides over SCE-PASEF without glyco-polygon filtering, at longer (90 min) and shorter (15 min) gradients, respectively. Early tests using the timsTOF Ultra 2 indicate even greater sensitivity and resolution.
Conclusion: The integration of glycan-specific polygon gating and SCE with PASEF on the timsTOF Pro platform, glyco-PASEF®, demonstrated substantial improvements in glycopeptide identification and characterization. Ongoing evaluations with the timsTOF Ultra 2 show even greater potential.

(P-164) Glycan Microarray Services and Bioinformatics through the National Center for Functional Glycomics (NCFG)

Jamie Heimburg-Molinaro[1], Akul Y. Mehta[1], Kelsey Charon[1], Sandra F. Cummings[1], Lauren Pepi[1], Richard D. Cummings[1]

[1]*Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School (HMS), Boston, MA,*

The National Center for Functional Glycomics (NCFG) at BIDMC at Harvard Medical School 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 to the scientific community for defining protein-glycan interactions. Our central discovery platform is glycan and glycopeptide microarrays, with glycans derived from a variety of sources, including chemo-enzymatic synthesis (*defined microarrays*), natural sources (*shotgun microarrays*), and microbially-derived components. A focused set of glycan components are in the process of being translated to the Luminex platform to allow for additional high-throughput 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 as a standard service- to date we have the method in place and can test on any sample type. We 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.

These programs are publicly accessible through the new NCFG website https://research.bidmc.org/ncfg/

A new and exciting development is that the archival data from the CFG glycan microarray that has been unavailable has now been restored and is available at: https://www.functionalglycomics.org/ under the control of the NCFG. The principal service offered through the NCFG is the analysis of all types of glycan binding proteins and biospecimens 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. Interconnected 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. Funding: *R24GM137763*.

(P-165) Proteomic and glycoproteomic biomarker discovery for chronic low dose ionizing radiation exposure in medaka fish

Sydney Bedillion[1], Marilyn Mason[2], Peng Zhao[1], Robert Bridger[1], Franklin E. Leach III[3], Ben Parrott[2], Olin E. Rhodes[2], Michael Tiemeyer[1], Lance Wells[1]

[1]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA,* [2]*Savannah River Ecology Lab, University of Georgia, Aiken, SC* [3]*Department of Chemistry, University of Georgia, Athens, GA,*

Human exposure to low dose ionizing radiation (LDIR) is inevitable, arising from both man-made and environmental sources. As these sources of LDIR such as medical imaging, occupational health hazards, and national security measures advance, the need to study the physiological effects and develop biomarkers of chronic exposure to LDIR is critical. However, many LDIR studies are

limited by the ability to rigorously monitor such low doses of ionizing radiation. The Low Dose Irradiation Facility (LoDIF) at the Savannah River Ecology Lab provides a unique capability for administering radiation exposure to aquatic organisms under environmentally relevant conditions. Using the LoDIF, we have begun to capture entire molecular profile changes associated with LDIR exposure through omic techniques. Here we present a proteomic and glycoproteomic perspective analysis of acute and chronic LDIR exposure in irradiated medaka fish livers.

Adult medaka fish (*Oryzias latipes*) were subject to a time course and dose variable radiation study. Medaka fish were exposed to an acute (14 days), chronic (70 days), or recovery (70 days + 14 day recovery) exposure at the LoDIF. Using sealed ^{137}Cs gamma ray irradiation sources, three doses of LDIR (approximately 5 mGy/day, 50 mGy/day, and 500 mGy/day) were administered with appropriately matched non-irradiated controls. Irradiated fish were euthanized and various organs were collected, snap frozen, and archived at −80 °C. For mass spectral analysis, irradiated livers were homogenized in organic solvents, yielding a precipitate of proteins and glycoproteins along with extracted lipids and soluble metabolites. Protein/glycoprotein preparations were proteolytically digested and analyzed by various combinations of LC-MS/MS methods optimized for molecular types using a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer. Untargeted proteomic and glycoproteomic data analysis was completed via Thermo Fisher Proteome Discoverer, Byonic, and open-source software platforms.

This work expands on proteomic and glycomic analysis previously published by our group that collected organs into sets and body regions and detected altered expression of 26 N-glycan structures and many proteins, that fell into 39 KEGG functional categories. Glycans modified by fucosylation and terminal sialic acid were determined to be the most responsive to LDIR exposure in this prior study. Additionally, proteomic analysis revealed changes in the immune system, endocrine system, and metabolism such as energy, carbohydrate, and lipid metabolism. The current study will allow higher tissue resolution assessment of LDIR. Molecular identifications are presented for each analysis through statistical tools and biological pathway enrichment analysis. Examining the changes in omic expression profiles through tissue-specific analysis will lead to LDIR biomarker identification and indicate possible mechanisms behind radiation-induced physiologic changes.

(P-166) Novel N-glycomics Analysis from Dried Blood Sample (DBS) Enables Remote Testing and Newborn Screening of CDGs

Earnest James Paul Daniel[2], Andrew C. Edmondson[2], Hudson H. Freeze[3], Michael Gelb[4], Miao He[1]

[1]Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, [2]Department of Pediatrics, Division of Human Genetics, The Children's Hospital of Philadelphia, Philadelphia, PA [3]Human Genetics Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, [4]Department of Biochemistry, University of Washington, Seattle, WA,

Congenital Disorders of Glycosylation (CDG) are a group of inborn errors of metabolism that result in multisystem disease due to defects in the biogenesis of glycoproteins or other glycoconjugates. Presently, there are over 190 known types of CDG, with more than 90 characterized by deficient N-linked protein glycosylation. Some CDG types, such as MPI-, SLC35C1-, and PGM1-CDG, can be effectively treated with monosaccharide therapy. Additionally, CDG types like SLC39A8-, SLC35A2-, and FUT8-CDG have shown partial responses to therapies involving manganese, galactose, and fucose, respectively. Emerging treatments for PMM2-CDG also highlight the importance of early diagnosis for optimal clinical outcomes. In this context, we present a robust method for screening CDG through N-glycomics analysis of glycoproteins extracted from dried blood samples (DBS).

We evaluated N-glycans released from dried blood samples (DBS) using a highly sensitive and accurate mass ESI-QTOF method. Semi-quantification of N-glycans was achieved using a C13-labeled glycopeptide to determine their abundance as a percentage of total glycans.

We have used 30 diagnostic plasma N-glycans, and the average recovery from DBS was 91%, with a range of 71%-113%. Imprecision studies performed on two-level controls from different punches of the same DBS card over 5 days showed a coefficient of variation between 3–17%. N-glycans remained stable in DBS kept at room temperature for over 4 months, with less than a 10% difference in quantification between storage at 4 °C and room temperature. Diagnostic N-glycan profiles for various type I and type II CDG, such as PMM2-, PGM1-, SLC35A2-, FUT8-, ATP6AP1-, and ALG3-CDG, from DBS were consistent with those from plasma analysis. Additionally, we analyzed newborn screening DBS punches of a 3-year-old child diagnosed with PMM2-CDG and successfully identified the diagnosis through a marked increase of mannose-deprived tetrasaccharide, along with increased Gal1GlcNAc2, Man3GlcNAc2, and Man4GlcNAc2, demonstrating the potential for diagnosing CDG at birth.

(P-167) Lipid Raft and Cell Surface Glycans-Mediated Signaling Contributing to the Maintenance of Pluripotency in Muse Cells

Yo Oguma[1], Yasumasa Kuroda[1], Mari Dezawa[1]

[1]*Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine,*

Cell surface glycans are utilized as stem cell markers, including stage-specific embryonic antigen (SSEA)-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Expression levels of these markers decrease or are lost as differentiation to somatic cells occurs, suggesting a close relationship between stem cell properties and these glycans. Muse cells, SSEA-3-positive endogenous pluripotent stem cells, are found in the connective tissue of nearly every organ, including the bone marrow and peripheral blood. To understand the basic properties of Muse cells, the function of SSEA-3 was analyzed. The SSEA-3 staining pattern is not homogeneously distributed on the cell membrane, appearing in spots. This suggests that SSEA-3 may localize within lipid raft microdomains on the cell membrane and modulate signaling pathways. In other words, SSEA-3 not only serves as a marker of Muse cells but also may play a role in maintaining their stemness. To examine this hypothesis, lipid rafts were isolated from Muse cells and other cell types with limited differentiation potential. The components of lipid rafts were comprehensively analyzed by mass spectrometry to identify activated signaling pathways via lipid rafts. Additionally, the possibility of modifying the cell surface with glycans and proteins identified through mass spectrometry to alter cell properties is being investigated. This research is expected to not only have biological significance in elucidating the functions of glycans and lipid rafts within cell surfaces but is also expected to be applied in developing new cell engineering mechanisms without gene induction.

(P-168) Establishing a curated human lectin collection for glycoprofiling

Karen P. Wang[1], Fred R. Chen[1], Ling Mao[1], Yi-Jing Chen[1], Jason T. Ku[1], Yi-Chang Liu[1]

[1]*GlycoGenetics, Inc., Taoyuan City, Taiwan,*

Human lectins have the biological roles in connecting the human glycome to physiological responses. However, the glycobiology field is still in need for a curated collection of recombinant human lectins dedicated for users to systematically probe glycoprofiles. We plan to establish a commercial source of such a collection of recombinant human lectins. We use recombinant expression systems to produce stable human lectins and subject these lectins to a collection of model human cell lines, each with a glycotransferase gene knocked out, to annotate each lectin's binding specificity in a genetically traceable manner. Here, we describe an example of using a collection of our recombinant galectins to probe cell lines carrying perturbed genes related to congenital disorders of glycosylation (CDG).

Session 8: Gaining Insights into Glycoenzymes and Glycoconjugates with: Artificial Intelligence/Machine Learning/Computer Science

(Key8-001) Are glycans really non-templated? Deploying AI deciphers how the underlying protein helps shape their glycans

Benjamin P. Kellman[1,2,3,4,5], Daniel Sandoval[6], Olga O. Zaytseva[7], Kelly Brock[8], Sabyasachi Baboo[9], Daniela Nachmanson[2,3,10], Edward B. Irvine[5,11], Erick Armingol[1,2,3], Nathan Mih[2,3], Yujie Zhang[1], Mia Jeffris[1,2], Philip Bartels[6], Thi Nguyen[6], Amy Tam[8], Sarah Gasman[12], Shlomi Ilan[5], Isaac Shamie[1,2,3], Jolene K. Diedrich[9], Xiaoning Wang[9], Esther van Woudenbergh[5], Meghan Altman[13], Anthony Aylward[2,3], Bokan Bao[1,2,3], Andrea Castro[2,3], James Sorrentino[1,2,3], Austin Chiang[1,2], Matt Campbell[14], Yannic Bartsch[5], Patricia Aguilar-Calvo[13,15,16], Christina Sigurdson[13,15], Galit Alter[5], Gordan Lauc[7], John R. Yates III[9], Debora Marks[8,17], Frederique Lisacek[18,19], Julien Mariethoz[18], Sigal Shaul[1,2], Mia Alteri[1,2], Daniel Bojar[20,21], Nathan E. Lewis[1,2,3]

[1]*Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA,* [2]*Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, USA* [3]*Bioinformatics and Systems Biology Graduate Program, University of California, San Diego, La Jolla, CA 92093, USA,* [4]*Augment Biologics, La Jolla, CA 92092,* [5]*Ragon Institute of MGH, MIT, and Harvard, Cambridge, MA, USA,* [6]*Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA,* [7]*Genos Glycoscience Research Laboratory, Borongajska 83H, Zagreb 10000, Croatia,*

[8]*Department of Systems Biology, Harvard Medical School, Boston, MA, USA,* [9]*Department of Molecular Medicine, The Scripps Research Institute, San Diego, California 92037, USA,* [10]*Moores Cancer Center, University of California San Diego, 3855 Health Science Drive, San Diego, CA, 92093, USA,* [11]*Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, USA,* [12]*Department of Pediatrics, Boston Medical Center, Boston, MA, USA,* [13]*Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, USA,* [14]*Institute for Glycomics, Griffith University, Queensland, Australia,* [15]*Department of Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, USA,* [16]*Department of Neurobiology, The University of Alabama at Birmingham, Birmingham, AL 35243,* [17]*Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142, USA,* [18]*Proteome Informatics Group, Swiss Institute of Bioinformatics, CH-1227 Geneva, Switzerland,* [19]*Computer Science Department & Section of Biology, University of Geneva, route de Drize 7, CH-1227, Geneva, Switzerland,* [20]*Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Gothenburg 41390, Sweden,* [21]*Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg 41390, Sweden,*

Glycosylation is ubiquitously described as non-templated. However, this contrasts with the observation that specific N-glycans are consistently placed at each site on many proteins. It is anticipated that the surrounding protein structure could constrain glycosylation, but how extensive is the influence of the surrounding protein? Based on extensive site-specific glycosylation data, we have developed an AI model that can predict the observed microheterogeneity. Here we used site-specific glycosylation data to we train a hybrid neural network to account for the proximal primary amino acid sequence (Long short-term memory recurrent neural network) and predict feasible N-glycosylation events using a graph neural network. This model, called the Interloping Saccharide Neural Network Extrapolation (InSaNNE) model, uses glycosite-flanking sequences to predict most human N-glycans documented in the GlyConnect database, along with changes in glycosylation seen from mutations in Spike of SARS-CoV-2 variants and IgG3 variants. This demonstrates that protein structure indeed acts as a template to help shape the feasible glycans at any given site in human proteins. With this information, we can now link hundreds of genetic variants associated with many diseases to candidate changes in glycan microheterogeneity induced by the mutations, such as in Oculocutaneous Albinism, Jakob-Creutzfeldt disease, Gerstmann-Straussler-Scheinker, and Gaucher's Disease, along with many glycan sites found on HIV ENV, and various monoclonal antibodies. Thus, not only does glycosylation have a sort of soft template in the in underlying protein sequence, but with the advent of powerful protein folding algorithms, we can now predict glycans from primary protein sequence, thereby completing glycoprotein structures for a wide range of applications.

(Key8-003) Integrative modeling of glycoproteins

Mateusz Sikora[1]

[1]*Malopolska Centre of Biotechnology, Krakow, Poland, Max Planck Institute of Biophysics, Frankfurt, Germany,*

Glycans, complex sugars covalently attached to proteins, play crucial roles in protein stability and function, participate in "self" recognition, and modulate protein-protein interactions. Unlike proteins, glycans typically do not form secondary structures and remain highly mobile, posing challenges for traditional structural biology techniques. This high mobility, coupled with glycan heterogeneity, complicates the elucidation of complete glycoprotein structures and hinders research on the role of glycans in protein function. Although computer simulations can help address these challenges, they often require millions of hours on specialized supercomputers, highlighting the need for approximate methods that can be integrated into protein-solving pipelines.

To overcome these obstacles, we developed a simplified, open-source method for rapidly predicting the span and shape of glycans with minimal computing power. Using this approach, we can accurately predict SARS-CoV-2 spike protein epitopes that are not shielded by glycans, as well as assess the impact of glycans on protein flexibility. We also demonstrate that this method can be integrated with structural biology methods and glycoproteomics pipelines, offering a comprehensive tool for glycoprotein analysis.

References

Turoňová B, Sikora M, Schürmann C, Hagen WJH, Welsch S, et al. In situ structural analysis of SARS-CoV-2 spike reveals flexibility mediated by three hinges. *Science*. 2020:**370**(6513).

Zhu R, Canena D, Sikora M, Klausberger M, Seferovic H, et al. Force-tuned avidity of spike variant-ACE2 interactions viewed on the single-molecule level. *Nat Comm*. 2022:**13**(7926).

Sikora M, von Bülow S, Blanc FEC, Gecht M, Covino R, Hummer G. Computational epitope map of SARS-CoV-2 spike protein. *PLoS computational biology*. 2021:**17**(4).

Tsai YX, Chang NE, Reuter K, Chang HT, Yang TJ, von Bülow S, Sehrawat V, Zerrouki N, Tuffery M, Gecht M, et al. Rapid simulation of glycoprotein structures by grafting and steric exclusion of glycan conformer libraries. *Cell*. **187**(5):1296–1311.e26.

(Key8-004) Glycoscience research and education in the AI era

Natarajan Kannan[1]

[1]*Dept. of Biochemistry & Mol. Biol. & Institute of Bioinformatics, University of Georgia, USA,*

Advances in Artificial Intelligence (AI) and machine learning models have accelerated the pace of research and education across multiple life sciences disciplines. However, the impact of these models in glycoscience research and education is yet to be fully realized. In this talk, I will describe our efforts to leverage advances in large language models for glycoenzyme research and education. Specifically, I will describe how representations (embeddings) generated from protein language models trained on a large corpus of sequence data can be used for alignment-free glycosyltransferase classification and function prediction. I will demonstrate that fine-tuning evolutionary scale models with labeled experimental datasets can significantly improve performance on downstream tasks such as predicting donor preference for understudied glycosyltransferases across the Tree of Life. The application of these models for generative design tasks as well as for training the next generation of glycoscientists will be described.

(PT-026) Evolutionary differentiation of Siglecs: insight into the binding specificity of Siglec-6 and Siglec-10 through all-atoms molecular dynamics simulations

Silvia D'Andrea[1], Ojas Singh[1], Elisa Fadda[2]

[1]*Department of Chemistry, Maynooth University, Maynooth, co Kildare, Ireland,* [2]*School of Biological Sciences, University of Southampton, Southampton, United Kingdom*

Sialic acid-binding immunoglobulin-type lectins (Siglecs) play a key role in regulating immune responses by recognizing specific sugar epitopes containing sialic acid (Neu5Ac). Siglecs are divided into two groups based on genetic similarity. Siglecs in the first group share 25–30% genetic similarity and include sialoadhesin, CD22, MAG, and Siglec-15, which are common in all mammals. The second group counts Siglecs related to CD33, with a genetic similarity of 50–99%, known for their rapid evolution. Despite their genetic differences, all Siglecs share a similar architecture and bind sialylated epitopes through a conserved Arg residue. Within this context, MAG (2nd group), Siglec-6 (1st group), and Siglec-11 (2nd group), represent a bit of a puzzling case, as mutation of the conserved sialic acid-binding Arg does not affect binding affinity. In this work we used molecular dynamics (MD) simulations to further investigate the matter by rebuilding an atomistic 3D model of the complex between Siglec-6 and GM1/2 epitopes embedded in a lipid bilayer. Our results show that recognition and binding of Siglec-6 to GM1/2 involves not only the evolutionarily conserved salt bridge between Arg122 and the GM1/2 sialic acid, but also a direct contact between the protein and the bilayer through insertion of the Trp127 sidechain and by a low-specificity electrostatic adhesion mediated by different Lys residues located on the Siglec-6 binding domain. This finding was confirmed by binding assays that show that the mutation of Trp127 abrogates binding. This molecular-level insight suggests that Siglecs may have evolved to select for specific epitopes by uniquely adapting their structure to complement the environment where these epitopes are found. As a term of comparison, we show the results of our preliminary analysis of Siglec-10, which binds different ganglioside epitopes (GT1B) to Siglec-6, as well as secreted sialylated glycans.

(PT-028) The Proteome as a Lectome: Predictions from Deep Learning Propose Substantial Protein-Carbohydrate Interplay

Samuel W. Canner[1], Ronald L. Schnaar[2,3], Jeffrey J. Gray[1,4]

[1]*Program in Molecular Biophysics, The Johns Hopkins University, Baltimore, MD, United States,* [2]*Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States* [3]*Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States,* [4]*Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, United States,*

A grand challenge to identify all the protein – carb interactions in organism. Experiments require extensive glycan arrays to definitively indicate non-binding, so computational screening of proteins for carbohydrate-binding provides a unique method. Recent computational techniques have focused primarily on predicting which protein residues interact with carbohydrates or which carbohydrate species a lectin binds to. Current computational estimates label 1.5∼10% of proteins as carbohydrate-binding proteins; however, 50–70% of proteins are known to be glycosylated, suggesting a potential wealth of proteins that

bind to carbohydrates. We therefore developed a novel dataset and neural network architecture, named Protein interaction of Carbohydrates Predictor (PiCaP), to predict whether a protein binds a non-covalent carbohydrate. We trained PiCaP on a dataset of known carbohydrate binders, and we selected proteins that we identified as likely *not* to bind carbohydrates, including DNA-binding transcription factors, cytoskeletal components, selected antibodies, and selected small molecule binding proteins. PiCAP currently achieves a 90% balanced accuracy (BACC) on protein-level predictions of carbohydrate binding/non-binding. Using the same dataset, we developed a model named CAPSIF2 to predict protein residues that interact with non-covalent carbohydrates. CAPSIF2 achieves a Dice score of 0.63 on residue-level predictions on our independent test dataset, outcompeting all previous models for this task. To demonstrate the biological applicablility of PiCAP and CAPSIF2, we investigated cell surface proteins of human neural cells (Schnaar 2024) and further predicted the likelihood of multiple proteomes to bind to carbohydrates and the binding interfaces of those proteins.

(PT-030) Predicting glycan structure from tandem mass spectrometry via deep learning

James Urban[1], Chunsheng Jin[2], Kristina A. Thomsson[2], Niclas G. Karlsson[3], Callum M. Ives[4], Elisa Fadda[5], Daniel Bojar[1]

[1]Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden, [2]Proteomics Core Facility at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden [3]Section of Pharmacy, Department of Life Sciences and Health, Faculty of Health Sciences, Oslo Metropolitan University, Oslo, Norway, [4]Department of Chemistry and Hamilton Institute, Maynooth University, Maynooth, Ireland, [5]School of Biological Sciences, University of Southampton, Southampton, UK,

Glycans constitute the most complicated post-translational modification, modulating protein activity in health and disease. However, structural annotation from tandem mass spectrometry (MS/MS) data is a bottleneck in glycomics, preventing high-throughput endeavors and relegating glycomics to a few experts. Trained on a newly curated set of 500,000 annotated MS/MS spectra, here we present CandyCrunch, a dilated residual neural network predicting glycan structure from raw liquid chromatography–MS/MS data in seconds (top-1 accuracy: 90.3%). We developed an open-access Python-based workflow of raw data conversion and prediction, followed by automated curation and fragment annotation, with predictions recapitulating and extending expert annotation. We demonstrate that this can be used for de novo annotation, diagnostic fragment identification and high-throughput glycomics. For maximum impact, this entire pipeline is tightly interlaced with our glycowork platform and can be easily tested at https://colab.research.google.com/github/BojarLab/CandyCrunch/blob/main/CandyCrunch.ipynb.

We envision CandyCrunch to democratize structural glycomics and the elucidation of biological roles of glycans.

(PT-032) Genetic control of N-linked Glycosylation through Protein Design

Meg Lunn-Halbert[1], Joel Allen[2], Rohith Krishna[1], Max Crispin[2], David Baker[1], Neil King[1]

[1]University of Washington, [2]University of Southampton

N-linked glycosylation is an important co- and post-translational modification of proteins that is often critical to correct protein folding and plays a crucial role in cell signaling. This process is sequential, beginning with the attachment of an oligomannose-type glycan to a nascent peptide strand as it enters the ER. The glycan is then trimmed and modified, resulting in a final glycoprotein displaying a heterogeneous population of complex glycans made of many different monosaccharides. Higher-order protein structure surrounding these glycosylation sites has been shown to heavily influence the degree of glycan processing on many viral glycoproteins such as HIV-1 envelope, SARS-CoV-2 spike, and the Lassa virus glycoprotein. Unlike typical healthy host glycoproteins that display complex glycans, these viral glycoproteins present incompletely processed oligomannose-type glycans at sterically hindered sites. This premature termination of processing occurs as the glycoprotein exits the folding cycle and the newly-folded structure surrounding the glycan blocks the preliminary mannose trimming steps to prevent any further processing. Here, we demonstrate how *de novo* protein design can play a key role in understanding this relationship between protein structure and glycosylation. The emergence of novel machine-learning methods that incorporate both protein and non-protein atoms such as RoseTTAFold diffusion All-Atom and AlphaFold3 have opened up the possibility for the design of *de novo* glycoproteins. By designing glycoproteins with steric restraint proximal to the glycosylation site, we show using protein structure alone we can genetically encode glycan composition and intentionally design oligomannose-type glycoproteins. We present a designed sterically-hindered oligomannose glycoprotein shown to have predominantly $Man_9GlcNAc_2$ glycans with a glycosylation profile similar to glycoproteins made in the presence of kifunensine, a small molecule inhibitor of mannosidase enzymes that prevents glycan processing. Systematically altering exposure of the glycan by truncating the designed protein led to predictable changes in glycosylation from high oligomannose-type to complex glycans. We also found that different structural motifs led to higher glycan occupancy rates, reinforcing previous findings that structural context influences both glycan occupancy and composition. Taken together, these data show that *de novo* protein design unlocks the way for controlling

site-specific glycosylation processing and occupancy. These new tools will be invaluable for studying the functional consequences of specific glycan moieties in processes such as cell adhesion, signaling, ligand binding, or immune pathway activation.

(P-169) An open-source framework for streamlining the model-based analysis of protein N-linked glycosylation kinetics

Konstantinos Flevaris[1], Pavlos Kotidis[2], Cleo Kontoravdi[1]

[1]Department of Chemical Engineering, Imperial College London, London SW7 2AZ, United Kingdom, [2]Present address: Biopharm Process Research, GSK, Stevenage, United Kingdom

In recent years, several models have been developed to successfully predict recombinant protein glycoprofiles in Chinese Hamster Ovary (CHO) cell cultures. However, their wide adoption within the bioprocessing community remains elusive due to the high level of domain knowledge required and limited accessibility. This work develops a novel end-to-end automated framework for the model-based analysis of glycosylation pathway kinetics, made available to experimentalists with minimal modeling experience. It aims to minimize manual decisions and account for uncertainty in parameterizing glycosylation kinetic models. In particular, a minimal network that can explain the observed glycoprofiles is generated. Given this information, a kinetic model of glycosylation is assembled in an automated way. This model extends the predictive capabilities of state-of-the-art mechanistic models to include the simultaneous prediction of the glycoprofiles of multiple proteins. Finally, the Golgi enzyme concentration levels are estimated via Bayesian inference. This framework is generically applicable to N-linked glycosylation and can be readily parameterized for the glycoproteins and cell systems of interest. A case study predicting glycoprofiles of recombinant IgG and host cell proteins (HCPs) in CHO cells reveals a considerable effect of HCPs glycosylation on the mAb glycoprofile. The proposed framework facilitates model-based insights into the effect of enzyme kinetics and their perturbations on experimentally measured glycoprofiles in biomanufacturing and clinical contexts.

(P-170) GlyCoDA: A compositional data analysis and domain adaptation perspective on disease risk prediction using plasma IgG N-glycans

Konstantinos Flevaris[1], Irena Trbojević-Akmačić[2], Gordan Lauc[2,3], Cleo Kontoravdi[1]

[1]Department of Chemical Engineering, Imperial College London, London SW7 2AZ, United Kingdom, [2]Genos Glycoscience Research Laboratory, Zagreb 10000, Croatia [3]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia,

Disease-specific biomarkers are crucial for managing chronic diseases through early diagnosis and screening. Plasma IgG N-glycans show promise as minimally invasive, modifiable biomarkers due to their plasticity in response to genetic and environmental factors. However, the analysis of glycomic datasets for biomarker discovery presents unique challenges: these datasets often comprise small to moderate sample sizes from demographically diverse cohorts and represent compositional data (CoDA), where each glycan is characterized by its relative abundance as part of a whole. To unlock the full biomarker potential of IgG glycomics, bespoke data analysis and machine learning (ML) techniques are required to develop reliable predictive tools for disease screening. This study proposes an ML-based N-glycomic analysis framework to develop classification models that capture disease-specific glycosylation variations while minimizing the influence of cohort-specific covariates. By coupling learnable log-contrast models with domain adaptation (DA) techniques, our framework aims to achieve robust generalization across cohorts with different characteristics. The proposed framework was designed and tested using inflammatory bowel disease (IBD) glycomic datasets from four cohorts. This open-source computational framework seeks to support the translation of glycan-based biomarkers into clinical applications.

(P-171) Development of Exchange System for Glycan Textual Notations to Integrate Various Glycan Databases and Improve Search Accuracy

Hiromitsu Shimoyama[1], Masaaki Matsubara[1], Issaku Yamada[1]

[1]The Noguchi Institute,

Various notation formats have been developed over the years to describe glycan structures, and then, databases with different notation exist independently. It is time-consuming to change the notation according to the databases to deposit and search

glycans. The purpose of our study is development of a program that automatically converts a glycan notation according to databases to reduce above tasks and enable us to access databases easily, accurately, and widely.

Each glycan notation has their own limitations, for example, IUPAC cannot to represent ambiguous linkage patterns. However, to access the database comprehensively and automatically, even such a structure should be kept internally. Therefore, Web3 Unique Representation of Carbohydrate Structures (WURCS), which can uniquely represent complex glycan structures, was chosen to design the internal objects. As WURCS is complicated, glycan structures are expected to be obtained in a simple way such as IUPAC and GlycoCT, and the program stores the input internally and converts it according to that of databases. For example, an information of queries with an ambiguous pattern obtained in GlycoCT format would be simplified for IUPAC databases but may be fully utilized for other databases. As the internal data object are repeatedly used for different interconversion, the object related code can be simplified (need not write similar code elsewhere). Then, such program would be easy to maintain.

(P-172) Glycomics Workbench: Harnessing the power of GenAI for Glycobiology research and education

Arun K. Datta[1]

[1]*National University, San Diego, CAUSA,*

Glycocodes are complex carbohydrate structures consisting of ten monosaccharides that encode important information for various biological processes in human including cell-cell interactions, extracellular signals, and cell differentiation. Deciphering glycocode requires understanding the sequence and linkage patterns of these monosaccharides or glycans. However, glycan analysis or Glycomics is more difficult than other -omics because it does not follow the linear flow of Central dogma. Rather, it needs meticulous and detailed analysis of glycosyltransferases and glycosidases involved in generating the glyco-codes. Recent developments in the machine learning technology, particularly GenAI, such as ChatGPT from OpenAI, Copilot from Microsoft, or Gemini from Google, has created an unprecedented opportunity that can tremendously help in such analysis and understanding the complexity involved. Besides these commercial tools, tools from open source, such as, Llama of Meta is also gaining popularity for developing scientific applications. In our developmental effort of Glycomics Workbench, this technology has been utilized for aiding glycoscience research and education. This author finds that a lot of glycocode related information can be obtained within minutes utilizing this technology that was earlier needed weeks if not months of manual curation. For the development of *Glycomics Workbench* portal that would be supported by the state-of-the-art Cyberinfrastructure (CI), we are utilizing Grid computing, Grid services, and data Grid. Grid technology offers multiple advantages including high scalability and Web accessibility. Grid computing provides accessibility to High-Performance Computing, such as, ACCESS of NSF. Grid services built on Open Grid Collaborating Environments (OGCE) are based on several Web service technologies. Our earlier work on CHOIS was built on OGCE. Data grid is a commodity grid that can host exabytes of data that has become essential for glycome analysis. Our earlier work on C-Grid development was to fulfill such a need. Moreover, a significant number of software tools and databases have been developed over the years for glycome analysis. However, utilization of some of those useful tools is restricted because those are not accessible via Web. This also restricts the semantic analysis of a vast amount of experimental data, such as, that were generated under the Consortium of Functional Glycomics (CFG). Our proposed Glycomics Workbench is designed to integrate such useful computational tools and resources for Glycomics that can better serve the Glyco-community. This presentation will demonstrate this technology and explain the fundamental aspect of GenAI and its various models. This presentation will also include the demonstration of Glycomics Workbench that utilizes this powerful technology for Glycome research and education.

(P-173) The Human Glycome Atlas (HGA) Project and TOHSA Knowledge-Base – Knowledge discovery by deciphering the information of Glycans

Achille Zappa[1], Sunmyoung Lee[1], Yukie Akune-Taylor[1], Kiyoko F. Aoki-Kinoshita[1,2]

[1]*Glycan and Life Systems Integration Center (GalSIC), Soka University, Hachioji, Japan,* [2]*Institute for Glyco-Core Research (iGCORE), Nagoya University, Nagoya, Japan*

The Human Glycome Atlas (HGA) Project, initiated in April 2023 as part of Japan's Large-scale Academic Frontier Promotion Project, aims to revolutionize glycoscience research by developing a comprehensive knowledge base called TOHSA (Total Human Glycome Structure Atlas). TOHSA is designed to be a semantic infrastructure that integrates and standardizes diverse glycan-related data, addressing the longstanding challenge of glycan structural complexity and data fragmentation in glycoscience.

TOHSA Semantic Knowledge-Base will comprehensively acquire information on all forms of glycans present in humans, the relationship between disease and glycans, and the mechanisms by which glycans are synthesized. These knowledges seek in

facilitating advancements in research and medical fields, and contributing to a true understanding of life mechanisms and the development of innovative treatments and preventive methods.

TOHSA advanced Semantic Web technologies, e.g. RDF, aim to create a unified platform for glycan information. This approach facilitates seamless integration of heterogeneous data sources, including glycan structures, glycoproteins, glycogenes, and associated diseases. Utilizing specialized ontologies such as GlycoRDF, alongside established life science ontologies and metadata, to ensure consistent and machine-readable data representation. Key feature of TOHSA is its implementation of controlled vocabularies and standardized metadata. These standardizations enable efficient data retrieval, cross-referencing, and interoperability with other life science databases.

The HGA project aims to catalog individual glycan structures along with associated clinical and phenotypic data, creating a comprehensive resource for glycoscience research. TOHSA's infrastructure is going to include user-friendly interfaces and visualization tools, allowing researchers to explore complex glycan pathways and relationships. The project will also incorporate Semantic & AI models for data analysis and prediction, enhancing its utility for knowledge discovery. Providing an integrated, standardized Semantic Knowledge-Base of glycome information, the HGA Project and TOHSA aim to accelerate glycoscience research, potentially leading to breakthroughs in understanding disease mechanisms and developing novel therapeutic approaches.

This initiative represents a significant step towards integrating glycomics with other omics fields, promising to unveil new insights into the roles of glycans in human biology and pathology. The Human Glycome Atlas and its TOHSA Knowledge-Base represent a leap forward in glycoscience research infrastructure. By offering an integrated, standardized, and friendly platform for glycan-related data, TOHSA addresses the challenge of integrating disparate human glycoscience data. To support this groundbreaking initiative, a comprehensive Data Management Plan (DMP) is put in place, outlining strategies, policies, and processes.

(P-174) Restoring Protein Glycosylation with GlycoShape

Ojas Singh[1], Callum M. Ives[1], Silvia D'Andrea[1], Carl A. Fogarty[1], Aoife M. Harbison[1], Akash Satheesan[2], Beatrice Tropea[2], Elisa Fadda[3]

[1]Department of Chemistry, Maynooth University, Maynooth, Ireland, [2]Hamilton Institute, Maynooth University, Maynooth, Ireland [3]School of Biological Sciences, University of Southampton, Southampton, SO17 1BJ, United Kingdom,

The field of structural biology has been transformed in recent years by ground-breaking innovations, enabling scientists to resolve the structure of biomolecules at the atomistic level of detail by cryogenic electron microscopy (cryo-EM) and to predict 3D structures from sequence by machine learning. Yet, none of these technologies can capture glycans, which remain largely invisible due to their inherent flexibility and heterogeneity, while impossible to predict due to lack of data to learn from. Here we introduce GlycoShape (https://glycoshape.org), an open access (OA) glycan structure database and toolbox designed to restore glycoproteins to their native and functional form in seconds. The GlycoShape database currently counts over 435 unique glycans, covering the human glycome and augmented by elements from a wide range of organisms, obtained from 1 ms of cumulative sampling from molecular dynamics (MD) simulations. These structures can be linked to proteins with a robust algorithm named Re-Glyco, built to evaluate steric complementarity with the protein landscape, and directly compatible with structural data in OA repositories, such as the RCSB PDB and AlphaFold Protein Structure Database, or own. The quality, performance, and broad applicability of GlycoShape is demonstrated by its ability to predict N-glycosylation occupancy, scoring a 93% agreement with experiment, based on screening all proteins in the PDB with a corresponding glycoproteomics profile, for a total of 4,259 N-glycosylation sequons.

(P-175) Direct & Automatic Alginate Oligosaccharide Mannuronic/Guluronic Acid Ratio Quantification from HCD-MS^2 Using Machine Learning

Xu Yang[1], Xinyi Ni[2], Ambrish Kumar[1], Yuefan Song[3], Jonathan Dordick[3], Christian Heiss[1], Pengyu Hong[2], Parastoo Azadi[1]

[1]Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA, [2]Department of Computer Science, Brandeis University, Waltham, MA, USA [3]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA,

Alginate is a versatile glycomaterial that could be used as a hydrogel-forming polymer or bioactive stimulant in oligosaccharide format. The linear backbone of alginate is made of isomeric α-L-guluronic acid (G) and β-D-mannuronic acid (M) building blocks, which are C-5 epimers. Since only G units can crosslink to each other by chelation with multi-valent ions, the accurate measurement of M/G ratio in an alginate sample is crucial for parameterizing its properties. However, except the classic high

temperature solution NMR method on alginate oligosaccharides, unambiguous, sensitive, and feasible analytical approaches are underdeveloped. We sought to utilize the quantitative hyperdimensional perception of the machine learning (ML) approach to directly quantify M/G ratios from higher collisional energy dissociation (HCD) MS^2 spectra of alginate oligosaccharides benchmarked by NMR. The average root-mean-squared-error (RMSE) on M/G ratio prediction could be achieved as low as 6.7% in cross-validation of formulated gradients of 3 kDa molecular weight cut-off (MWCO) M-rich (83% M/17% G) and G-rich (12% M/88% G) alginate mixtures. It turns out that to prepare alginate oligosaccharides with precisely defined M/G ratio and well characterized degree of polymerization (DP) is the major challenge we have faced. We have developed a homogeneous mild acid depolymerization condition to reduce the average DP of G-rich (25% M/75% G) alginate segments without introducing other structural changes. Ongoing efforts are focused on separating precisely depolymerized alginate oligosaccharides by DP. We hope the refined ML model can serve as a more sensitive, complementing method to NMR of studying alginate fine structure. 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).

(P-176) Implementation and Expansion of Archetypes in GlyTouCan

Akihiro Fujita[1], Thomas Masding[2], Kiyoko F. Aoki-Kinoshita[1,2,3]

[1]Institute for Glyco-core Research (iGCORE), Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan, [2]Graduate School of Science and Engineering, Soka University, Tokyo, Japan [3]Glycan & Life System Integration Center, Soka University, Tokyo, Japan,

GlyTouCan [1] now contains over 35,000 nearly identical glycan structures. Such glycans are defined as having the same structure but different reducing end, such as the examples, G70733VZ and G92561HL which are the alpha anomer and beta anomer respectively. Moreover the mentioned structural similarities can make such glycans particularly difficult to distinguish adding complexity to search and glycan analysis. This study defined the archetype glycan concept [2], which has a more inclusive reducing end structure, as such is able to act as a unified representation of said reducing ends. For example, archetype glycan G96225KT is able to represent both mentioned glycans G70733VZ and G92561HL. As such this approach allows us to better organize these highly similar glycan structures within GlyTouCan.

Many archetype glycans have now been registered in GlyTouCan, and we have expanded this concept to provide a more comprehensive organization of glycan structures. For example in this research, we have demonstrated that the archetype glycan concept can also be applied to nearly identical monosaccharide composition structures. These compositions are composed of the same monosaccharides; however, may differ in that the monosaccharides for example have differences in anomercity or are in an open chain form.

This effort has been supported by WURCS [3], a glycan computational descriptor which can describe a wide range of glycan structures. The implementation of archetype glycans in GlyTouCan has improved search functionality and data organization. As of the latest pre-release (October 2023), 59,224 archetype glycans were identified from 211,328 total glycans. This new functionality has been integrated into the glycan list and entry pages in GlyTouCan, and its full release on the next version of GlyTouCan v4 is underway.

References

Fujita A et al. *Nucleic acids research*. 2021:**49**(D1):D1529–D1533. doi: https://doi.org/10.1093/nar/gkaa947.
https://doc.glytoucan.org/.
Matsubara M et al. *Journal of chemical information and modeling*. 2017:**57**(4):632–637. doi: 10.1021/acs.jcim.6b00650.

(P-177) Glydentify, a deep learning tool for classifying glycosyltransferase function

Aarya Venkat[1], Ruili Fang[2], Zhongliang Zhou[2], Shaan Gill[1], Natarajan Kannan[1]

[1]Department of Biochemistry and Molecular Biology, University of Georgia, [2]Department of Computer Science, University of Georgia

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 that uses protein language models for the classification of glycosyltransferases (GTs) and donor prediction. 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% accuracy. The tool also predicts GT-A donor binding preferences with an accuracy of 91%. Notably, Glydentify identifies key residues that contribute to a prediction, thereby adding an explainable 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 GT research. The application is freely available on GitHub and can be accessed directly through any web browser (https://huggingface.co/spaces/arikat/Glydentify).

(P-178) New features and data in the GlyGen knowledgebase

Sujeet Kulkarni[1], Rene Ranzinger[1], 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

To advance its goal of democratizing glycoscience and enhancing the reach of glycosylation concepts, GlyGen continues to add new features, species, and data types to the knowledgebase. By maintaining existing collaborations with large international database providers like EBI, NCBI, PDB, and GlyCosmos and establishing new collaborations with EMBL, the University of Zagreb, and Proteomic Data Commons, we have built and continue to upgrade our comprehensive knowledgebase and web portal (https://www.glygen.org) for glycoscience.

In the last year, GlyGen has added several new species (yeast, slime mold, pig, and chicken) and their associated glycans and proteins to the knowledgebase. To further develop understanding of the role of glycosylation in disease, GlyGen has worked on integrating biomarker data into the knowledgebase. To facilitate access to this information, GlyGen has developed biomarker search and information pages and cross-linked the biomarker data with glycan, protein, motif, and publication information on the GlyGen web-based interface. All integrated datasets are also available on the GlyGen bioinformatics data portal, the GlyGen RESTful webservice-based APIs, and a SPARQL endpoint to allow programmatic access. GlyGen has also added new features that enhance user interactions. The Glycan Feature Viewer is a new interactive tool that displays glycan sequence in SNFG notation and allows users to highlight residues of interest based on residue type, motifs, and enzymatic activities. GlyGen 3D Viewer integrates Mol* Viewer in GlyGen to display three-dimensional glycan structures generated by GLYCAM and protein structures from PDB and AlphaFold.

GlyGen also holds in-person and online workshops and webinars to facilitate interactions with the glycoscience community. User feedback is highly valued and helps direct the development of new features and incorporation of new data across GlyGen release cycles. Our goal is to provide a central glycoscience knowledgebase that allows easy access to complex cross-domain information that describes the biology of glycans and glycoproteins. To schedule an individual demo of GlyGen or add your data to GlyGen, contact Sujeet Kulkarni (sujeet.kulkarni25@uga.edu).

(P-179) Protein structure, a genetic encoding for glycosylation

Benjamin P. Kellman[1,2,3,4,5,*], Daniel Sandoval[6], Olga O. Zaytseva[7], Kelly Brock[8], Sabyasachi Baboo[9], Daniela Nachmanson[2,3,10], Edward B. Irvine[5,11], Erick Armingol[1,2,3], Nathan Mih[2,3], Yujie Zhang[1], Mia Jeffris[1,2], Philip Bartels[6], Thi Nguyen[6], Amy Tam[8], Sarah Gasman[12], Shlomi Ilan[5], Isaac Shamie[1,2,3], Jolene K. Diedrich[9], Xiaoning Wang[9], Esther van Woudenbergh[5], Meghan Altman[13], Anthony Aylward[2,3], Bokan Bao[1,2,3], Andrea Castro[2,3], James Sorrentino[1,2,3], Austin Chiang[1,2], Matt Campbell[14], Yannic Bartsch[5], Patricia Aguilar-Calvo[13,15,16], Christina Sigurdson[13,15], Galit Alter[5], Gordan Lauc[7], John R. Yates III[9], Debora Marks[8,17], Frederique Lisacek[18,19], Nathan E. Lewis[1,2,3,*]

[1]Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA, [2]Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, USA [3]Bioinformatics and Systems Biology Graduate Program, University of California, San Diego, La Jolla, CA 92093, USA, [4]Augment Biologics, La Jolla, CA 92092, [5]Ragon Institute of MGH, MIT, and Harvard, Cambridge, MA, USA, [6]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA, [7]Genos Glycoscience Research Laboratory, Borongajska 83H, Zagreb 10000, Croatia, [8]Department of Systems Biology, Harvard Medical School, Boston, MA, USA, [9]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California 92037, United States, [10]Moores Cancer Center, University of California San Diego, 3855 Health Science Drive, San Diego, CA, 92093, USA, [11]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, USA, [12]Department of Pediatrics, Boston Medical Center, Boston, MA, USA, [13]Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, USA, [14]Institute for Glycomics, Griffith University, Queensland, Australia, [15]Department of Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, USA, [16]Department of Neurobiology, The University of Alabama at Birmingham, Birmingham, AL 3524, [17]Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142, United States, [18]Proteome Informatics Group, Swiss Institute of Bioinformatics, CH-1227 Geneva, Switzerland, [19]Computer Science Department & Section of Biology, University of Geneva, route de Drize 7, CH-1227, Geneva, Switzerland,

DNA, RNA, and proteins are synthesized using template molecules, but glycosylation is not believed to be constrained by a template. However, if cellular environment is the only determinant of glycosylation, all sites should receive the same glycans on average. This template-free assertion is inconsistent with observations of microheterogeneity—wherein each site receives distinct and reproducible glycan structures. Here, we test the assumption of template-free glycan biosynthesis. Through structural analysis of site-specific glycosylation data, we find protein-sequence and structural features that predict specific glycan features. To quantify these relationships, we present a new amino acid substitution matrix that describes "glycoimpact" – how glycosylation varies with protein structure. High-glycoimpact amino acids co-evolve with glycosites, and glycoimpact is high when estimates of amino acid conservation and variant pathogenicity diverge. We report hundreds of disease variants near glycosites with high-glycoimpact, including several with known links to aberrant glycosylation (e.g., Oculocutaneous Albinism, Jakob-Creutzfeldt disease, Gerstmann-Straussler-Scheinker, and Gaucher's Disease). Finally, we validate glycoimpact quantification by studying oligomannose-complex glycan ratios on HIV ENV, differential sialylation on IgG3 Fc, differential glycosylation on SARS-CoV-2 Spike, and fucose-modulated function of a tuberculosis monoclonal antibody. In all, we show glycan biosynthesis is accurately guided by specific, genetically-encoded rules, and this presents a plausible refutation to the assumption of template-free glycosylation.

(P-180) Inferring Tissue and Cell-type Glycosyltransferase Specificity from Single-Cell Gene Expression Data

Nathan Edwards[1], Abdullah Abdul[1]

[1]*Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center,*

While many of the glycosyltransferases that synthesize cellular glycans are ubiquitously observed across tissues and cell-types, some glycosyltransferases are only expressed in specific contexts, potentially resulting in context-specific glycan structures. Furthermore, while the attachment of a specific residue with specific linkage can often be carried out by multiple enzymes, these enzymes may have context specific activity, introducing a more nuanced context-dependent effect on glycan structures. We explore the extent to which we can (re-)discover tissue and cell-type glycosyltransferase specificity by studying tissue specific gene expression and cell-type and tissue specific single-cell gene expression data.

For each glycosyltransferase, we assess whether the expression values can reliably distinguish, using logistic regression, the samples or cells from a specific tissue or cell-type. The ability to distinguish is calibrated by measuring the recall at 90% specificity under 10-fold cross validation for each GT and comparing this performance with that of randomly chosen genes. We apply this pipeline to 115 human GTs from the GlyGen GlycoTree Sandbox project, representing 42 different residue attachment activities, and the tissue-specific bulk gene expression data, with 17K samples and 54 tissues types, and tissue and cell-type-specific single-nuclei gene expression data, with 209K nuclei profiles from 8 tissues and 43 cell-types, available from the Adult GTEx project.

Unsurprisingly, glycotransferases responsible for common N-linked glycan structural motifs, such as the N-glycan core or antennae residues show little tissue-specific or cell-type specific expression. Our strongest specificity results, however, are for sialic acid, fucose, and sulfate transferases, suggesting that the decoration and capping of mature glycan structures is where tissue and cell-type specificity is most exercised. We hope that as this computational pipeline matures it will facilitate a context-dependent understanding of glycosyltransferase activity and the functional annotation of glycan structural motifs.

(P-181) Annotating Glycans with Functions to Facilitate Expanded Connectivity of Glycoscience Knowledge

Michael Tiemeyer[1], René Ranzinger[1], Jeet Vora[2], Mindy Porterfield[1], Nathan Edwards[3], Raja Mazumder[2], The Glycan Function Annotation Consortium[4]

[1]*The Complex Carbohydrate Research Center, University of Georgia,* [2]*Department of Biochemistry and Molecular Medicine, The George Washington University School of Medicine and Health Sciences* [3]*Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center,* [4]*Multi-institutional and international participants in the 1st and 2 nd "Workshops on Glycan Function Annotation",*

Dynamic changes in protein and lipid glycosylation impact protein homeostasis, membrane functions, development, inflammation, immunity, aging, neural function, pathogen interactions, and almost all aspects of human health from fertilization to death. However, current bioinformatic resources that capture disease and phenotype information focus primarily on the macromolecules represented within the central dogma of molecular biology (DNA, RNA, proteins). In order to gain a more complete understanding of biological processes and especially disease, there is a need to capture the functional impact of glycans and glycosylation. To that end, two "***Workshops on Glycan Function Annotation***" were organized by GlyGen (www.glygen.org) with the purpose of supporting ongoing biocuration efforts to annotate glycan functions such that they can be connected to

diverse data types and knowledgebases. Together, these workshops established an understanding of what kinds of glycoscience data are currently captured in existing resources and built a foundation for establishing an ontology that describes the hierarchical relationships between function, glycan structure, and biological context. Workshop participants from bioinformatic resources proposed solutions for bridging perceived gaps and described areas where curators, data wranglers, and text mining experts could collaborate. They also highlighted the need for guidance from glycoscience experts to establish function vocabularies. In response, workshop participants with glycoscience knowledge proposed a high-level set of function classes that captures a broad swath of glycan activities. The results of these workshops will be presented in more detail and a pathway for biocuration and glycoscience community engagement will be proposed.

(P-182) Automated Glycan Binding Analysis: Probability Modeling of Glycan Structure Abundance from Glycan Binding Data

Zachary Klamer[1], Anna Repesh[1], Hoang-Le Tran[1], Nick Berning[2], Xi Chen[2], Jian Zhang[2], Brian Haab[1]

[1]Van Andel Institute, Grand Rapids, Michigan, USA, [2]ZBiotech, Aurora, Colorado, USA

The most readily available method to quantify glycosylation of a sample is by probing the sample with glycan binding proteins. These glycan binding assays, such as lectin arrays, only require a small amount of sample, yet can provide specific measurements of the monosaccharide epitopes driving glycan mediated interactions, thereby providing data relevant for both research and clinical use. However, the results of such assays are largely opaque to non-glycobiologists due to the current inability to provide automated and accurate interpretation of the data. To make glycobiology more accessible, we have developed an analysis software which uses glycan binding affinity to model the relationship between binding observations and estimates the abundance of the glycan structures. Drawing from the abundance of published glycan array data, the algorithm can estimate the apparent binding kinetics between glycans and proteins, while also considering publication frequency as to constrain results to biologically probable solutions. It can additionally integrate orthogonal glycan data, such as glycosyltransferase abundance or mass spectrometry data. This approach is flexible enough to be used with any glycan binding data and can analyze samples from any biological system where the range of possible glycans is known. We have tested this software with both purified proteins and clinical samples, resulting in the successful identification of N- to O- glycan ratios, N-Glycan branching, termini type, and isomeric presentation. Additionally, when evaluating transferrin, a simple glycoprotein, we achieved exact prediction of the predominant glycan. This software transforms the use and interpretation of glycan binding data, ultimately rendering glycobiology more accessible to researchers and clinicians.

(P-183) GlycoBERT and GlycoBART: Large language models for glycan structure prediction from tandem mass spectrometry

Rudiyanto Gunawan[1], Ejas Althaf Abtheen[1], Changyou Chen[2], Sriram Neelamegham[1]

[1]Department of Chemical and Biological Engineering, University at Buffalo-SUNY, Buffalo 14260, [2]Department of Computer Science, University at Buffalo-SUNY, Buffalo 14260

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is commonly used for the structural analysis of glycans. The outputs from such experiments include precursor mass-over-charge (m/z), LC retention time, and MS/MS fragmentation spectra. It is challenging to translate these data into glycan structures due to the complexity in analyzing glycan isomers which commonly have overlapping fragment ions with varying intensity. The current paper describes the development of deep-learning transformer-based large language models (LLMs) for the analysis of MS/MS spectra. Specifically, it describes glycoBERT, built on the BERT (Bidirectional Encoder Representations from Transformers) architecture, for glycan classification from MS/MS spectra. It also describes glycoBART, built on BART (Bidirectional Auto-Regressive Transformers)—a variant of BERT—for glycan structure generation tasks. Our presentation will describe a novel tokenization strategy to transform MS data into a 'sentence' that is used as input to these transformer-based models. Besides peaks, information related to experimental settings, relative retention time, and precursor mass are included in the 'MS sentence'. A custom vocabulary for mass spectra was constructed using the recently published compendium of MS/MS glycomics, called CandyCrunch (Nat Methods, 2024;21(7):1206–1215). Finally, a tokenizer was developed to generate MS sentences as inputs for training and inference using the transformer architecture. GlycoBERT was formulated for multiclass classification task, assigning a mass spectrum to one of 3307 possible glycan structures. Meanwhile, GlycoBART was designed for conditional sequence inference, generating glycan structure predictions as sequences of glycan antennae. When trained using CandyCrunch dataset, GlycoBERT achieved 95.2% test accuracy, outperforming CandyCrunch model (87.7%). While GlycoBART reached 93% accuracy, the method has the capability for *de novo* inference, generating predictions of glycan structures not present in the training data. This generative

capability is a significant advantage over previous computational methods. In an application to an independent MS/MS dataset from Glycoforest (Anal Chem. 2017; 89(20):10932–10940), GlycoBART was able to produce glycan structures with matching topologies and compositions with expert-labeled ground truth, achieving 4 correct predictions out of 7 *de novo* glycan inference tasks.

(P-184) A Platform for Disseminating ML-Ready Datasets for Insights into Glycoconjugates and Glycoenzymes

Urnisha Bhuiyan[1], GlyGen Team Members[1,2]

[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*

Advancements in glycoanalytical technologies have generated a vast array of glycomics and glycoproteomics datasets that require significant data processing. GlyGen's machine learning (ML)-ready datasets alleviate this burden by being pre-processed and systematically organized. These datasets need only minimal adjustments and allow users with basic domain knowledge to efficiently develop ML models. By providing well-structured data, ML-ready datasets enable scientists to focus on model development and analysis. This can empower researchers to uncover new glycan biomarkers, elucidate glycan-mediated disease mechanisms, and accelerate the development of glycan-based therapeutics.

GlyGen has integrated and made available glycomics and glycoproteomics ML-ready datasets for disease risk assessment for conditions like Type II Diabetes and Clear Cell Renal Carcinoma. Additionally, GlyGen is actively developing glycoproteomic datasets to predict glycosylation and phosphorylation sites using existing data, as well as glycomic datasets aimed at predicting treatment response outcomes for conditions such as prediabetes, cancer, and many others. These initiatives are supported by our established collaborations with leading international database providers, including EBI, NCBI, PDB, and GlyCosmos, along with new partnerships with EMBL, the University of Zagreb, and Proteomic Data Commons. Through these collaborations, we continue to enhance our comprehensive glycoscience knowledgebase and web portal, available at https://www.glygen.org.

We perform thorough literature searches using resources such as PubMed and Web of Science to identify publicly available glycome and glycoproteome datasets. These datasets undergo additional data processing and cleaning to ensure they meet GlyGen's ML-readiness standards before integration into our resource, where information for each dataset is provided on our wiki page https://wiki.glygen.org/ML-Ready_Datasets.

Glycobiology has greatly advanced through ML in areas such as structure prediction, biomarker discovery, and systems biology. GlyGen seeks to build on these advancements by offering user-friendly tools and resources, such as ML-ready datasets, that simplify advanced data analysis, making it accessible to researchers across various technical backgrounds. Our objective is to empower a broader range of researchers to utilize ML in their work, thereby accelerating discoveries and progress in the field. GlyGen also provides the infrastructure through BioCompute Objects for the community to submit their ML-ready datasets for distribution, fostering new collaborations and enhancing model transparency through explainable AI.

(P-185) Towards An Automated Pipeline for Glycoprotein Simulations in Martini Coarse-grained Model

Alam Ahmad Hidayat[1,2], Mateusz Sikora[1,2]

[1]*Malopolska Center for Biotechnology, Jagiellonian University, Krakow, Poland,* [2]*Max Planck Institute for Biophysics, Frankfurt, Germany*

Most proteins undergo a modification where complex sugar molecules (glycans) are covalently attached. However, glycans exhibit large conformational freedom, making them difficult subjects for structural biology and resulting in a poor understanding of their role in protein mechanics. Classical molecular dynamics simulations (MDS) offer a method to examine how glycans affect proteins at ns-μs time scales, which can be insufficient to sample larger protein motions or interactions. Utilizing coarse-grained (CG) frameworks such as Martini has been overwhelmingly successful in extending the time and spatial scales in biomolecular simulations. However, parameters for only a handful of common glycan species are present in Martini [1], [2], and a systematic approach for preparing a glycosylated system in CG is not yet clear. In this project, we are aiming to develop a standardized pipeline to prepare glycosylated protein systems in Martini simulations, including automated parametrization of glycans. By using the GlycoSHIELD glycan library [3] as an atomistic reference and conformer selection tool, we are currently developing a simple software to assist in systematically parametrizing the glycans and to rapidly generate their CG models, while providing clash-free starting structures. Here, we start with introducing parameters of N-glycosylated residues in Martini 3 force field and preparing glycoproteins for CG simulations as a test case.