# PROGRAM AND ABSTRACTS FOR 2017 ANNUAL MEETING OF THE SOCIETY FOR GLYCOBIOLOGY

**November 5–8, 2017**
Portland, Oregon, USA

# SFG MEETING 2017

*Sunday November 5, 2017*

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| 9 am-1 pm | Glycoprotein Technologies Satellite |
| Chair: | Parastoo Azadi (CCRC/University of Georgia) |
| 9 am-3 pm | Trainee Mentoring Program |
| Chairs: | Lance Wells (CCRC/University of Georgia) |
| | Karen Colley (University of Illinois at Chicago) |
| 10 am-5 pm | Bioinformatics Satellite |
| Chair: | Rene Ranzinger (CCRC/University of Georgia) |
| 3:30-5:00 pm | Board of Directors Meeting |

**Session 1. Meyer and Kornfeld Awards Lectures**

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| Chair: | Karen Colley (University of Illinois at Chicago) |
| 5:30-5:45 | Opening Remarks |
| 5:45-6:30 | Karl Meyer Award Lecture |
| | Jamey Marth (University of California, Santa Barbara) |
| 6:30-7:15 | Rosalind Kornfeld Award Lecture |
| | Gillian Air (University of Oklahoma) |
| 7:30-9:30 | Reception |

*Monday November 6, 2017*

**Session 2. Glycans in metabolic regulation and development (8:30-10:00 am)**

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| Chair: | Kelley Moremen (CCRC/University of Georgia) |
| 8:30-9:00 | Heparan sulfate in lipid and iron homeostasis. |
| | Jeff Esko (University of California, San Diego) and Maura Poli (University of Brescia, Brescia, Italy) |
| 9:00-9:20 | Studying atypical dystroglycanopathies using zebrafish models. |
| | Chiara Manzini (George Washington University) |
| 9:20-9:50 | Cell-specific regulation and roles of O-GlcNAc: key to understanding brain function. |
| | Jerry Hart (Johns Hopkins) and Olof Lagerlöf (Karolinska Institute). |
| 9:50-9:55 | Poster talk: Structures of human O-GlcNAcase and its complexes reveal a new substrate recognition mode. |
| | Jiaoyang Jiang (University of Wisconsin-Madison) |
| 9:55-10:00 | Poster talk: Dynamic splicing of a glycosyltransferase modulates enzyme activity and secretory granule morphology. |
| | Leslie Revoredo (NIH/NIDCR, Developmental Glycobiology Section) |

Coffee (10:00-10:30 am)

**Session 3: Glycan biosynthesis and function (10:30 am-12:25 pm)**

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| Chair: | Nancy Dahms (Medical College of Wisconsin) |
| 10:30-11:00 | Stress, glycomics, and disease-A combined perspective. |
| | Stuart Haslam (Imperial College London) and Matthew Shoulders (MIT) |
| 11:00-11:05 | Poster talk: Siglec-8 Is an activating receptor on human eosinophils mediating integrin-dependent adhesion, ROS generation and apoptosis. |
| | Daniela J. Carroll (Northwestern University Feinberg School of Medicine) |

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| 11:05-11:25 | Orchestration of mucin type O-glycosylation by the multiple activities of the ppGalNAc-T family of transferases.<br>Tom Gerken (Case Western Reserve University) |
| 11:25-11:45 | Targeting site-specific O-glycosylation for novel therapeutics.<br>Adam Linstedt (Carnegie Mellon University) |
| 11:45-11:50 | Poster talk: Bone marrow macrophage galectin-3 regulates platelet production through recognition of O-glycans on megakaryocytes.<br>Melissa M. Lee-Sundlov (Blood Research Institute, BloodCenter Wisconsin and the Department of Hematology, Brigham & Women's Hospital & Harvard Medical School) |
| 11:50-12:20 | Structural and functional perspectives on the glycan-mediated tuning of Notch activity.<br>Robert Haltiwanger (University of Georgia/CCRC) and Vincent Luca (Moffitt Cancer Center) |
| 12:20-12:25 | Poster talk: C-mannosylation of thrombospondin repeats.<br>Hans Bakker (Hannover Medical School) |
| 12:25-12:30 | Poster talk: Mapping sites and molecular functions of O-glycosylation.<br>Katrine T. Schjoldager (University of Copenhagen) |
| 12:30-1:30 | Lunch on your own |
| 1:30-4:00 | Poster Session I |
| 3:00-3:55 | NIH Listens. Discussion with NIH Program Staff. Pamela Marino (NIH) |

**Session 4: Glycan related diseases and disorders I (4:00-5:30 pm)**

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| Chair: | Susan Bellis |
| 4:00-4:30 | Genetic and pharmacologic regulation of the oligosaccharyltransferase.<br>Reid Gilmore (University of Massachusetts Medical School) and Joe Contessa (Yale University) |
| 4:30-4:35 | Poster talk: Anti-inflammatory functions of Siglec-E and Siglec-9 and alteration of their ligands in mouse airway inflammation and lung emphysema.<br>Zhou Zhu (Yale University School of Medicine) |
| 4:35-4:55 | Glycans and their binding proteins in ocular surface disease.<br>Pablo Argüeso (Schepens Eye Research Institute/Massachusetts Eye and Ear, Harvard). |
| 4:55-5:00 | Poster talk: Regulatory functions of heparan sulfate in prostate stem/progenitor cell activities and prostatic tumorigenesis.<br>Lianchun Wang (University of Georgia, CCRC) |
| 5:00-5:20 | Mucin-type O-glycans in intestinal mucus barrier homeostasis and disease.<br>Lijun Xia (Oklahoma Medical Research Foundation) |
| 5:20-5:25 | Poster talk: IgA Nephropathy: An autoimmune kidney disease involving the clustered O-glycans of IgA1 as autoantigens.<br>Matthew B. Renfrow (University of Alabama at Birmingham) |
| 5:25-5:30 | Poster talk: A semantic approach to Molecular Glycophenotype classification for disease diagnostics.<br>Jean-Philippe F. Gourdine (Oregon Health and Science University and Undiagnosed Diseases Network) |

**Session 5. Innovator Award Lecture** (5:30-6:30)

*Tuesday November 7, 2017*

**Session 6: Glycolipids in health and disease (8:30-10:00 am)**

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| Chair: | Anant Menon (Weill Medical College/Cornell University) |
| 8:30-8:55 | ABC transporters as glucosyl ceramide flippases in glycosphingolipid biosynthesis.<br>Clifford Lingwood (The Hospital for Sick Children, University of Toronto) |

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| 8:55-9:20 | Transport of lipopolysaccharides across the bacterial cell envelope.<br>Natividad Ruiz (The Ohio State University) |
| 9:20-9:45 | Identifying lipid scramblases for dolichol-based glycolipids involved in protein *N*-glycosylation in the endoplasmic reticulum.<br>Anant Menon (Weill Medical College, Cornell University) |
| 9:45-9:55 | Poster talk: A new hypothesis for Lec5.<br>Mark Lehrman (UT Southwestern Medical Center) |
| 9:55-10:00 | Poster talk: LmeA, a periplasmic membrane-bound protein, is critical for lipomannan biosynthesis and cell envelope integrity in mycobacteria.<br>Yasu S. Morita, Sarah Osman, Kathryn C. Rahlwes (University of Massachusetts, Amherst) |
| 10:00-10:30 | Coffee |

**Session 7: Glycans in pathogenesis and infection (10:30-12:15)**

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|---|---|
| Chair: | Christopher West (University of Georgia) |
| 10:30-10:55 | Making home sweet and sturdy: Investigations into the roles of glycosylation in the cyst wall of Toxoplasma gondii.<br>Louis Weiss (Albert Einstein College of Medicine) |
| 10:55-11:10 | Elucidating glycomic contributions to Toxoplasma biology and virulence.<br>Christopher West (University of Georgia) |
| 11:10-11:15 | Poster talk: Neoglycoproteins as biomarkers for cutaneous leishmaniasis.<br>Katja Michael (University of Texas at El Paso) |
| 11:15-11:20 | Poster talk: Identifying the in vitro Arginine-GlcNAcylation targets of the NleB/SseK family of effectors.<br>Nicholas E. Scott (University of Melbourne) |
| 11:20-11:40 | Active roles for heparan sulfonated proteoglycans and growth factors in human papillomavirus infectious entry: A Trojan horse mechanism.<br>Michelle Ozbun (University of New Mexico) |
| 11:40-11:45 | Poster talk: Changes in cell surface glycans in women with bacterial vaginosis and impact on Fusobacterium vaginal colonization.<br>Kavita Agarwal (Washington University in St. Louis) |
| 11:45-11:50 | Poster talk: Antibody fucosylation restricts Fc gamma receptor IIIA (CD16A) N-glycan motion to reduce affinity.<br>Dan Falconer (Iowa State University) |
| 11:50-12:10 | Sialic acid structures and linkages - variation within animals and effects on virus interactions<br>Colin Parrish (Cornell University College of Veterinary Medicine) |
| 12:10-12:15 | Poster talk: Inhibition of O-glycan biosynthesis using hexosamine analogs.<br>Sriram Neelamegham (State University of New York Buffalo) |
| 12:15-1:30 | Lunch on your own |
| 1:30-4:00 | Poster Session II |
| 4:00-4:45 | Business Meeting |

**Session 8. MCP and Glycobiology Significant Achievement Award Lectures (4:45-6:15)**

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|---|---|
| Chair: | Kelley Moremen (President Elect) |
| 4:45-5:30 | Molecular and Cellular Proteomics Award Lecture (5 min intro, 40 min talk)<br>Stuart Haslam (Imperial College London) |
| 5:30-6:15 | Glycobiology Significant Achievement Award Lecture (5 min intro, 40 min talk)<br>H. Jafar Nejad (Baylor College of Medicine) |
| 6:15-7:00 | Break |
| 7:00-11:00 | Banquet |

*Wednesday November 8, 2017*

**Session 9: Expect the unexpected from microbes (8:30-9:50 am)**

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| Chair: | Christine Szymanski (CCRC/University of Georgia) |
| 8:30-9:00 | New insights into glycoconjugate receptors for cholera toxin.<br>Jennifer Kohler (UT Southwestern Medical Center)<br>Ulf Yrlid (University of Gothenburg, Sweden) |
| 9:00-9:05 | Poster talk: Understanding influenza A specificity: An evoluation of paradigms.<br>Robert J. Woods (CCRC/University of Georgia). |
| 9:05-9:25 | Unique features of the machinery that pathogenic and commensal microbes use to attack host glycans.<br>Alisdair Boraston (University of Victoria). |
| 9:25-9:30 | Poster talk: Nascent microbiome and early metabolism are perturbed by pre- and post-natal exposure to artificial sweeteners.<br>Stephanie Oliver-Van Stichelen (NIH/NIDDK). |
| 9:30-9:50 | Who will win? The endless battle between campylobacters and bacteriophages in the gut.<br>Christine Szymanski (CCRC/University of Georgia). |
| Coffee (9:50-10:15 am) | |

**Session 10: Glycoengineering and glycan related therapeutics (10:15-11:45)**

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|---|---|
| Chair: | Don Jarvis (University of Wyoming) |
| 10:15-10:35 | Plant based glycan engineering for the production of therapeutic proteins.<br>Herta Steinkellner (Universität für Bodenkultur Wien) |
| 10:35-10:55 | Rapid mapping of glycoprotein structure-activity relationships by shotgun scanning glycomutagenesis.<br>Matthew DeLisa (Cornell University) |
| 10:55-11:15 | Developing anti-inflammatory drugs targeting selectins.<br>Richard Cummings (Beth Israel Deaconess Medical Center, Harvard Medical School) |
| 11:15-11:45 | Lawrence Tabak (NIH) News from the NIH: Information and Q&A. |
| 11:45-1:00 | Lunch on your own |

**Session 11: Glycan related diseases and disorders II (1:00-2:30 pm)**

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| Chair: | Stuart Haslam |
| 1:00-1:30 | Mutations in the gene for O-GlcNAc transferase are causal for X-linked intellectual disability.<br>Lance Wells (CCRC/University of Georgia)<br>Charles Schwartz (Greenwood Genetic Center) |
| 1:30-1:50 | Aberrant glycosylation in breast cancer results in modulation of the immune micro-environment.<br>Joy Burchell (Kings College London) |
| 1:50-2:10 | The consequences of human ganglioside deficiency.<br>Michael Tiemeyer (CCRC/University of Georgia) |
| 2:10-2:20 | Poster talk: A family of carbohydrate tumor antigens with a proposed common mechanism of action.<br>Fred Brewer (Albert Einstein College of Medicine) |
| 2:20-2:25 | Poster talk: Fut2 is required for methacholine-induced airway hyperreactivity in a mouse model of allergic asthma.<br>Dorota S. Raclawska (University of Colorado Denver) |
| Closing remarks: | 2:25-2:40. |

# SFG 2017 POSTER PROGRAM

PS1= Poster session 1 PS2=Poster session 2

## Session 1: Glycans in metabolic regulation and development

**Session 2: Glycan biosynthesis and function**

**Session 3: Glycan related diseases and disorders**

**Session 4: Glycolipids in health and disease**

**Session 5: Glycans in pathogenesis and infection**

**Session 6: Expect the unexpected from microbes**

**Session 7: Glycoengineering and glycan related therapeutics**

### (1) Heparan Sulfate in Lipid and Iron Homeostasis

Maura Poli[1], Ferdous Anower-E-Khuda[2], Philip L.S.M. Gordts[2,3], Michela Asperti[1], Paolo Arosio[1] and Jeffrey D. Esko[2]

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

Prior studies have established an important role for hepatic heparan sulfate proteoglycans in lipid homeostasis. The evidence shows that syndecan-1, a transmembrane proteoglycan, acts as an endocytic receptor on hepatocytes for chylomicron and VLDL remnant lipoprotein particles in the circulation. The activity of syndecan-1 depends on the interaction of the heparan sulfate chains with apolipoproteins E and AV present on the remnant lipoprotein particles. Structure-function studies in Hep3B cell mutants prepared by CRISPR/Cas9 and *in vivo* using recombinant AAV vectors have elucidated molecular features of syndecan-1 essential for lipoprotein binding and uptake. In parallel to these studies, we found that hepatocyte heparan sulfate also plays a crucial role in iron homeostasis. Iron is an essential micronutrient for all organisms because it acts as a cofactor in various biological processes; however, excess iron is toxic because it can catalyze the formation of aggressive reactive oxygen species that promote membrane, protein and DNA oxidation leading to cell death. Thus a tight regulation of free iron is needed both at systemic and at the cellular level. Iron uptake and release involves the regulated expression of the peptide hormone hepcidin, which negatively controls cellular iron export by binding to ferroportin and facilitating its turnover. Hepcidin is mainly expressed in the liver under the control of the BMP6/SMAD1/5/8 pathway. Heparin is a strong inhibitor of this pathway and of hepcidin expression *in vitro* and *in vivo*, suggesting that BMP6/SMAD pathway could involve endogenous heparan sulfate. This conclusion is supported by pharmacological and genetic studies in Hep3B cells and *in vivo* in heparan sulfate deficient mice. Thus, hepatocyte heparan sulfate proteoglycans coordinate both lipid and iron metabolism, but by very different mechanisms. Whether these two systems are coordinated under stress conditions is a topic for further discussion.

### (2) Studying atypical dystroglycanopathies using zebrafish models

M. Chiara Manzini

*Institute for Neuroscience, George Washington University*

Interactions between glycoproteins on the cell surface and the extracellular matrixhave a vital role in maintaining visceral or musculoskeletal tissue structure, and loss of these interactions by disrupting glycosylation leads to a spectrum of pediatric diseases affecting multiple organsand resulting in severe cognitive and motor impairment

Our work focuses on a group of autosomal recessive diseases, termed dystroglycanopathies, where the transmembrane glycoprotein alpha-dystroglycancannot interact with its extracellular ligands. Dystroglycanopathies lead to profound developmental delay and early childhood mortality and comprise the most severe forms of congenital muscular dystrophy (CMD), which are usually associated with cerebral and ocular malformations. Gene identification efforts in the past decade discovered that many genes mutated in dystroglycanopathies are glycosyltransferases assembling an array of O-linked glycans necessary for alpha-dystroglycan binding to its extracellular ligands. More recentgene discovery has identified atypical cases where mutated genes are shared across multiple glycosylation disorders and/or expand the spectrum of disease.

Here, I will focus on recent studies from my group exploring these atypical dystroglycanopathy genes and on how modeling human mutations in the zebrafish has sped up both gene discovery and functional studies. We have explored how dolichol-phosphoryl-mannose synthase (DPMS) which synthesizes dolichol-phosphate-mannose, the principal donor of mannose in the endoplasmic reticulum (ER), is functioning in the brain and muscle. In addition, we have recently found that mutations in the ER inositol phosphataseinositol polyphosphate 5-phosphatase K (INPP5K)cause a novel CMD syndrome with intellectual disability and reduced dystroglycan glycosylation. We are exploring whether trafficking of glycoproteins, instead of glycosylation, could be altered in this disorder.

### (3) Cell-specific regulation and roles of O-GlcNAc: key to understanding brain function

Olof Lagerlöf[1] and Gerald W. Hart[2]

*[1]Department of Neuroscience, Karolinska Institutet; [2]Department of Biological Chemistry, Johns Hopkins University*

Many proteins in the brain are modified by β-N-acetylglucosamine on the hydroxyl group of serines and threonines (O-GlcNAc). Unlike complex O- and N-linked glycoconjugates found on the outside of cells and in the extracellular matrix, O-GlcNAc is typically not further elongated and expressed in the cytoplasm, nucleus and mitochondria. While O-GlcNAc is ubiquitous in animal cells, the brain is one of the organs where it is the most abundant. Animal models and mutations discovered in human patients with intellectual disability have shown O-GlcNAc to be critical for the development of the central nervous system. The role of O-GlcNAc for adult brain function has remained elusive. New data from ours and other laboratories suggest that O-GlcNAc plays distinct roles in different types of neurons. We have discovered recently that αCaMKII neurons in the paraventricular nucleus (PVN) of the hypothalamus become activated upon food intake. Once activated, they turn off further intake. Fasting decreases and glucose stimulates O-GlcNAc incorporation in these, but not in neighboring αCaMKII-negative, cells. The feeding-induced activation of

the αCaMKII PVN cells depends on O-GlcNAc transferase (OGT), the enzyme that attaches O-GlcNAc to proteins. When removing OGT selectively from αCaMKII PVN neurons in adult mice, the animals rapidly grew obese due to overeating. OGT regulates the function of the αCaMKII PVN neurons, at least in part, by maintaining their number of excitatory synapses, the cell-cell junctions by which neurons receive stimulatory input. New data indicate that OGT regulates excitatory synapse number by maintaining a major isoform of the excitatory glutamate receptor, the GluA2/3 AMPA receptor, at synapses. Others have demonstrated that in hypothalamic Agrp neurons, on the other hand, O-GlcNAc is increased by starvation and important for energy expenditure through the Kcnq3 potassium channel. In this talk, O-GlcNAc is discussed as a key mechanism by which the brain controls energy homeostasis. The regulation and function of O-GlcNAc, however, diverges between different neuronal circuits. Supported by NIH P01HL107153, R01GM116891, R01DK61671, N01-HV-00240, Diabetes Wellness Research & Wellness Foundation and Stiftelsen Sigurd & Elsa Goljes Minne. *Dr. Hart receives a share of royalty received by the universityon sales of the CTD 110.6 antibody, which are managed by JHU.*

### (4) Structures of human O-GlcNAcase and its complexes reveal a new substrate recognition mode

Jiaoyang Jiang
*School of Pharmacy, University of Wisconsin-Madison*

Human O-GlcNAcase (hOGA) is the unique enzyme responsible for the hydrolysis of the O-linked beta-N-acetyl glucosamine (O-GlcNAc) modification, an essential protein glycosylation event that modulates the function of numerous cellular proteins in response to nutrients and stress. Here we report crystal structures of a truncated hOGA, which comprises the catalytic and stalk domains, in apo form, in complex with an inhibitor, and in complex with a glycopeptide substrate. We found that hOGA forms an unusual arm-in-arm homodimer in which the catalytic domain of one monomer is covered by the stalk domain of the sister monomer to create a substrate-binding cleft. Notably, the residues on the cleft surface afford extensive interactions with the peptide substrate in a recognition mode that is distinct from that of its bacterial homologs. These structures represent the first model of eukaryotic enzymes in the glycoside hydrolase 84 (GH84) family and provide a crucial starting point for understanding the substrate specificity of hOGA, which regulates a broad range of biological and pathological processes.

### (5) Dynamic splicing of a glycosyltransferase modulates enzyme activity and secretory granule morphology

Suena Ji[1,2], Leslie Revoredo[1,2], Adina L. Milac[3], Duy T. Tran[1,2] and Kelly G. Ten Hagen[1,2]
[1]*NIH/NIDCR;* [2]*Developmental Glycobiology Section;* [3]*Institute of Biochemistry of the Romanian Academy*

Mucins are highly glycosylated proteins with unique structural and rheological properties that mediate adhesion, hydration and the protection of biological surfaces. Mucins are glycosylated by a family of enzymes (PGANTs in*Drosophila*) in the Golgi apparatus and packaged into secretory granules before being delivered to the cell surface or the extracellular matrix. Here, we identify a novel member of the*pgant*family that undergoes tissue-specific differential splicing to modulate secretory granule morphology. Interestingly, splicing of this subdomain confers unique substrate specificity, allowing the complete glycosylation of a cargo mucin. In the absence of this splice variant, the mucin is not fully glycosylated and secretory granules take on an irregular, shard-like appearance. Our study provides the first evidence that alternative splicing can alter PGANT substrate specificity and that the glycosylation status of mucin cargo can affect secretory granule morphology.

### (6) Stress, Glycomics, and Disease-A Combined Perspective

Stuart M. Haslam[1] and Matthew D. Shoulders[2]
[1]*Department of Life Sciences, Imperial College London, UK;* [2]*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA*

The endoplasmic reticulum's (ER's) unfolded protein response (UPR) up-regulates chaperones and quality control mechanisms in response to protein misfolding stress. Surprisingly, we recently discovered a new function of the UPR, in modulating N-glycan maturation of N-glycosylated glycoproteins. We will demonstrate that the UPR's XBP1s transcription factor is responsible for modifying the N-glycan profile at both the global cellular level and at the individual glycoprotein level. Characterisation of changing N-glycosylation profiles was achieved by the application of MALDI-TOF based glycomic methodologies. This previously unknown capacity of the UPR to modulate N-glycan maturation identifies an unanticipated link between protein misfolding stress responses and the molecular architecture of extracellular N-glycoproteins. This could have implications for numerous biological systems such as aging and disease processes plus the control of glycosylation of recombinant glycoproteins.

### (7) SIGLEC-8 IS AN ACTIVATING RECEPTOR ON HUMAN EOSINOPHILS MEDIATING INTEGRIN-DEPENDENT ADHESION, ROS GENERATION AND APOPTOSIS

Daniela J. Carroll[1], Jeremy A. O'Sullivan[1], David B. Nix[2], Yun Cao[1], Michael Tiemeyer[2] and Bruce S. Bochner[1]
[1]*Department of Medicine, Division of Allergy and Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL;* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA*

Eosinophils are major effector cells in diseases including asthma, rhinitis, certain gastrointestinal disorders, and atopic dermatitis. Current treatments include mediator antagonists and anti-inflammatory drugs that reduce allergic cell numbers

and inhibit mediator release, but they are not fully effective or curative. Siglecs (sialic acid-binding, immunoglobulin-like lectins) are type I transmembrane proteins expressed primarily on leukocytes. Among them is Siglec-8, a CD33 subfamily member that is selectively expressed on the cell surface of human eosinophils. Siglec-8 has an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), putatively responsible for signal transduction. Engagement of Siglec-8 causes resting eosinophil apoptosis in a caspase-dependent manner. In cytokine-activated eosinophils, Siglec-8 binding causes caspase-independent apoptosis with increased mitochondrial damage and ROS production, but exact signaling mechanisms are unknown.

Using a mAb or a multimeric synthetic sulfated sialoglycan ligand recognizing Siglec-8, we first examined Siglec-8-mediated phosphorylation of signaling molecules in the presence or absence of IL-5 priming using phosphoproteomics analysis. In follow-up experiments, we used blocking antibodies, pharmacological inhibitors and western blot analysis to fully understand the necessity of the identified phosphoproteins for Siglec-8 function in human eosinophils.

We observed that Siglec-8 engagement on cytokine-primed eosinophils promoted rapid β2-integrin dependent eosinophil spreading and adhesion, and that β2-integrin dependent adhesion was necessary for subsequent ROS generation and apoptosis. Furthermore, analysis of the eosinophil phosphoproteome using LC-MS/MS identified 237 proteins that were differentially phosphorylated following Siglec-8 engagement and some of these proteins were linked to pathways associated with cell adhesion and cytoskeleton organization. Additional experiments demonstrated that Siglec-8-mediated ROS was generated via NADPH oxidase activation, because pretreatment of eosinophils with catalase (a superoxide scavenger) or NSC23766 (a Rac GTPase inhibitor) completely inhibited Siglec-8-mediated eosinophil apoptosis at $IC_{90}$ concentrations of 400 – 1000 U/mL and 40 μM respectively. Finally, engagement of Siglec-8 on cytokine-primed eosinophils resulted in increased phosphorylation of Akt, p38 and JNK1 that was also β2-integrin dependent, and pharmacologic inhibition of these pathways using LY294002 (a PI3K/Akt inhibitor), SB203580 (a p38 inhibitor) or SP600125 (a JNK1/2 inhibitor) prevented Siglec-8-mediated eosinophil apoptosis at $IC_{50}$ concentrations of 1 μM, 1.1 μM and 4.3 μM respectively.

In conclusion, we demonstrate for the first time that Siglec-8 functions as an activating receptor on cytokine-primed eosinophils via positive regulation of β2-integrins, NADPH oxidase and a subset of protein kinases.

### (8) Orchestration of Mucin Type O-Glycosylation by the Multiple Activities of the ppGalNAc-T Family of Transferases

Thomas A. Gerken
*Depts of Biochemistry, Pediatrics and Chemistry, Case Western Reserve Univ. Cleveland OH*

A large family of mammalian UDP-GalNAc:polypeptide GalNAc transferases (ppGalNAc-T's) initiate and define sites of mucin type O-glycosylation by adding GalNAc onto Ser and Thr residues of target substrates. Family members are further classified into peptide and glycopeptide preferring subfamilies based on their preferred substrates. Structurally, most isoforms contain an N-terminal catalytic domain linked to a C-terminal carbohydrate binding lectin domain whose roles in modulating peptide and glycopeptide specificity are just being understood. Several disease states including cancers are linked to the aberrant expression of individual ppGalNAc-T isoforms, hence the need to fully understand and characterize individual isoform specificity.

Our earlier studies utilizing random peptide substrates have shown that the ppGalNAc-T isoforms possess both unique and overlapping peptide substrate specificites which can be used for predicting isoform specific sites of O-glycosyaltion (http://isoglyp.utep.edu). We have now further characterized the different rates of glycosylation of Thr vs Ser acceptors for nearly half of the ppGalNAc-T isoforms, observing significant differences (2 to 13 fold) depending on isoform. These data will further improve our ability to predict isoform specific sites of glycosylation.

We have also shown that the ppGalNAc-Ts can present two types of activities against GalNAc-O-Ser/Thr containing glycopeptides. These are termed remote and neighboring glycosylation, which are dependent on the transferase lectin and catalytic domains, respectively. While the former effect directs O-glycosylation to acceptor sites located 6-~17 residues from a previous Thr/Ser-O-GalNAc (in an N or C terminal direction) the latter recognizes prior glycosylation sites at the -1, + 1 or + 3 positions relative to the acceptor site, depending on isoform. In both cases, the presence of a prior site of glycosylation enhances the rate of glycosylation on the acceptor site.

With these studies we now have a better understanding of this transferase family, where each isoform possess a unique combination of catalytic domain and lectin domain peptide and glycopeptide specificity. This suggests that these transferases may operate in an ordered process to perform their glycosylation functions, where one transferase likely produces an optimal glycopeptide substrate for the next. Taken together this suggests that mucin type O-glycosylation is potentially highly orchestrated by the cell. Furthermore, the demonstration of multiple activities for these transferases, suggests that in the analysis of the role of ppGalNAc-Ts linked to disease, an examination of all of their possible activities must be considered.

Supported by NIH grants GM113534 and CA78834.

1. 2017Gerken, T. et.al. 2011. J. Biol. Chem. 286:14493–14507.
2. Revoredo. L et. al. 2016. Glycobiology 26, 360–376.

### (9) Cell-based screening identifies drugs targeting Golgi-based O-glycosylation

Adam D. Linstedt and Lina Song
*Dept. Biol. Sci., Carnegie Mellon, Pittsburgh PA*

Although there are currently none, drug-like modulators of the large enzyme family that initiates site-specific O-glycosylation in the Golgi complex (UDP-N-acetyl-α-D-galactosamine polypeptide N-acetylgalactosaminyltransferases or ppGalNAc-Ts) hold promise as therapeutics for major diseases such as chronic kidney disease and cancer. To enable high-throughput screening for drug-like inhibitors, we developed cell-based fluorescent sensors of O-glycosylation mediated by ppGalNAc-T isozymes. Among the first hits, we identified a compound that: a) specifically acts on the T3 sensor, b) specifically inhibits purified ppGalNAc-T3 in an *in vitro* assay, c) appears to be non-toxic, d) does not alter ppGalNAc-T cellular expression or localization, and e) does not perturb overall O-linked or N-linked glycosylation as determined using lectin staining. The inhibitor binds ppGalNAc-T3 with low micromolar affinity conferring a non-competitive/mixed mode of inhibition. Significantly, it enhances proteolysis of FGF23 both in cultured cells and after intraperitoneal injection of mice. In the latter case, the level of the proteolyzed, inhibitory, C-terminal FGF23 fragment increases by 8-fold showing the promise of the compound to antagonize aberrant FGF23 signaling in chronic kidney disease.

### (10) Bone marrow macrophage galectin-3 regulates platelet production through recognition of O-glycans on megakaryocytes

Melissa M. Lee-Sundlov[1,2], Renata Grozovsky[2], Silvia Giannini[2], Haley E. Ramsey[3], Ulla Mandel[4], Martha Sola-Visner[3] and Karin M. Hoffmeister[1,2]

*[1]Blood Research Institute, BloodCenter Wisconsin; [2]Department of Hematology, Brigham & Women's Hospital & Harvard Medical School; [3]Division of Newborn Medicine, Boston Children's Hospital; [4]Copenhagen Center for Glycomics, University of Copenhagen*

Circulating platelet count, an essential indicator of health and disease, has been intimately linked with platelet glycosylation. Desialylated platelets bind to hepatic receptors or lectins, specifically the Ashwell-Morell receptor the macrophage aMß2 integrin, leading to their clearance. The role of lectins and glycans in platelet production (thrombopoiesis), specifically in the regulation of bone marrow (BM) platelet precursors, megakaryocytes (MKs), is understudied.

We report a novel role for BM macrophage galectin-3 in regulating MKs expressing the Thomsen-Friedenreich (TF) antigen, a tumor-associated molecule. The TF antigen is a cryptic disaccharide on O-glycans usually covered by a sialic acid moiety added by the sialyltransferase ST3Gal1. To investigate the role of the TF antigen in thrombopoiesis, we generated ST3Gal1$^{MK\text{-}KO}$ mice with increased TF antigen specifically in the MK lineage (Cre-Lox recombination system with PF4-Cre expression). Increased TF antigen expression on ST3Gal1$^{MK\text{-}KO}$ MKs was verified by peanut agglutinin (PNA) binding. Other blood cell lineages had no increase in TF antigen expression. ST3Gal1$^{MK\text{-}KO}$ mice developed significant thrombocytopenia, but had normal platelet clearance, suggesting that TF antigen affected BM thrombopoiesis rather than platelet survival. Immunostaining with an anti-TF antigen antibody showed that TF antigen-expressing MK progenitors were significantly increased in ST3Gal1$^{MK\text{-}KO}$ mice. Despite this, ST3Gal1$^{MK\text{-}KO}$ mice did not have more MKs. *In vitro* megakaryocyte maturation and proplatelet production were normal in primary BM cells isolated from ST3Gal1$^{MK\text{-}KO}$ mice, pointing to extrinsic factors in the ST3Gal1$^{MK\text{-}KO}$ BM environment affecting the MK production.

ST3Gal1$^{MK\text{-}KO}$ BM smears revealed increased hemophagocytosis, indicative of an increase in phagocytic macrophages. *In vivo* macrophage ablation by injection of clodronate liposomes significantly reduced the numbers of macrophages, resulting in increased BM MK counts, and normalized platelet counts. ST3Gal1$^{MK\text{-}KO}$ BM cells shows an increase in mRNA expression of galectin-3, a macrophage marker and a ligand for the TF antigen. Mice lacking galectin-3 alone had increased MK numbers and normal platelet counts. When ST3Gal1$^{MK\text{-}KO}$ mice were crossed with galectin-3$^{KO}$ mice, MK progenitors and MK numbers were significantly increased compared to control and ST3Gal1$^{MK\text{-}KO}$. Moreover, immunostaining of ST3Gal1$^{MK\text{-}KO}$ BM sections show co-localization of galectin-3 macrophages and MKs.

The data support the notion that interaction of MK O-glycans and macrophages expressing galectin-3 play a role in thrombopoiesis. Although macrophage galectin-3 appears to play a minor role in normal thrombopoiesis, increased TF antigen expression on MKs in pathological conditions can cause activation of macrophage galectin-3 leading to inhibition of MK maturation and subsequent thrombocytopenia.

### (11) Structural and functional perspectives on the glycan-mediated tuning of Notch activity

Vincent C. Luca[1] and Robert S. Haltiwanger[2]

*[1]Department of Drug Discovery, Moffitt Cancer Center & Research Institute, Tampa, FL USA; [2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA USA*

The Notch receptor is an ancient glycoprotein that guides development in all metazoans. Defects in Notch signaling result in a wide variety of developmental disorders and cancers in humans; thus, a detailed understanding of the mechanisms by which Notch is activated and regulated is essential to the development of therapeutics. Notch is extensively modified on its 36 EGF repeats with *O*-fucose, *O*-glucose and *O*-GlcNAc glycans, which function to modulate both protein stability and binding to cognate ligands Delta-like (DLL) and Jagged. Furthermore, the elongation of Notch *O*-fucose moieties by Fringe ß3-*N*-acetylglucosaminyltransferases acts as a ligand 'selectivity switch' that inhibits responsiveness to Jagged and potentiates activation by DLL. Here, we highlight a series of collaborative studies dissecting the structural, biophysical and cellular mechanisms by which glycans fine-tune Notch signaling activity.

To capture intrinsically low-affinity Notch-ligand complexes for structural studies, we used *in vitro* evolution to introduce affinity-enhancing mutations into DLL4 and Jagged1. This strategy enabled us to determine co-crystal structures of the interacting domains of Notch1-DLL4 and Notch1-Jagged1. The structures reveal detailed molecular information about the Notch-ligand binding interface, including a direct role for *O*-fucose modifications on Notch1 EGF repeats 8 and 12 in ligand engagement. Replacement of the fucose on EGF8 with a fucose analog interferes with binding to DLL but not Jagged ligands, suggesting that fucose analogs can be used as DLL-specific Notch inhibitors. Elimination of the sites on EGF repeats 8 and 12 by mutagenesis confirms their importance in binding to both Delta and Jagged ligands, and additional experiments indicate that Fringe modification of these sites is crucial for enhancement of Delta-mediated Notch1 ligand-binding and activation. Surprisingly, Fringe modifications at sites outside of the ligand-binding domains (EGF repeats 6 and 36) play key roles in the inhibition of Jagged1-mediated Notch1 activation by a yet unknown mechanism. Taken together, these studies illuminate the remarkable complexity by which Notch activity is regulated by tissue-specific glycosylation. This work was supported by NIH GM061126 (RSH) and by 4R00CA204738-02 (VCL).

### (12) C-mannosylation of thrombospondin repeats

Aleksandra Shcherbakova, Birgit Tiemann, Falk FR Buettner and Hans Bakker

*Institute of Clinical Biochemistry, Hannover Medical School, Germany*

Protein C-mannosylation is the only known type of glycosylation involving a C-C linkage. The canonical reaction is the modification of the first tryptophan of a WxxW motif. C-mannosyltransferases use Dol-P-Man as donor substrate. Although C-mannosylation is known for over twenty years now, its function is still poorly understood. Since we identified *C. elegans* DPY-19 as a C-mannosyltransferase in 2013, new experimental possibilities are available. Surprisingly, four DPY-19 homologs were found to be present in mammals, of which one was just found to be mutated in the most common form of globozoospermia, which results in infertility. This protein (DPY19L2) is expressed exclusively in sperm and, although expected to be a C-mannosyltransferase, no activity has been attributed to it. We could, however, show that mammalian DPY19L1 and DPY19L3 are C-mannosyltransferases having different specificities. This was established by generating CRISPR-Cas knockouts in CHO cells of the different putative C-mannosyltransferases, both single and multiple, resulting in cells with reduced or no C-mannosylation. We could show that the thrombospondin repeats of mouse UNC5A netrin receptor, containing elongated C-mannosylation motifs (WxxWxxWxxC), could be mannosylated on all three tryptophans and that DPY19L1 was modifying the first two tryptophans, whereas DPY19L3 specificity mannosylated the third tryptophan, thereby not using the established WxxW motif as substrate. First concrete aspects of the function of C-mannosylation will be presented as well. By direct comparison of C-mannosylated and non-mannosylated UNC-5, C-mannosylation was shown to be able to significantly increase protein secretion and also to greatly increase the stability of the protein.

### (13) Genetic and Pharmacologic Regulation of the Oligosaccharyltransferase

Marta Baro[2], Natalia A. Cherepanova[1], Cecilia Lopez-Sambrooks[2], Natalie Rinis[2], Shiteshu Shrimal[1], Reid Gilmore[1] and Joseph N. Contessa[2]

[1]*Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School;* [2]*Department of Therapeutic Radiology, Yale School of Medicine*

Metazoans express two oligosaccharyltransferases (OSTs) that are composed of a catalytic subunit (STT3A or STT3B), six shared subunits, and complex-specific accessory subunits. Point mutations in the human STT3A and STT3B genes cause severe forms of congenital disorders of glycosylation, indicating that both OSTs are essential for normal human health and development. The STT3A complex cotranslationally scans the nascent polypeptide for sequons as the nascent protein enters the ER lumen through the protein translocation channel. The interaction between the STT3A complex and the translocon is mediated by the DC2 and KCP2 subunits of the STT3A complex. The STT3B complex glycosylates sequons that have been skipped by STT3A. We have used quantitative glycoproteomics to identify classes of sequons that are hypoglycosylated in STT3A(-/-) or STT3B(-/-) cells. Quantitative data was obtained for roughly 1000 glycosylation sites. Glycosylation sites that are most severely affected by loss of the STT3A complex include NXS sequons with sub-optimal flanking residues. Sequons within small cysteine-rich protein domains also exhibited STT3A dependence. STT3B dependent glycosylation sites included extreme C-terminal sequons, and sites located within small extracellular loops of polytopic integral membrane proteins. Surprisingly, roughly 5% of acceptor sites showed increased glycosylation in STT3A(-/-) cells including multiple cryptic sequons in the lumenal ER chaperone GPP94.

Redundancy of the OST in human cells raised the possibility that OST activity could be regulated by small molecules. A novel N-glycosylation inhibitor (NGI-1) was identified through a HTS campaign using a cell-based bioluminescent reporter. NGI-1 reduces glycosylation of both STT3A and STT3B dependent substrates, and STT3B sites are more sensitive to this inhibitor. Using STT3A(-/-) and STT3B(-/-) cells we show that NGI-1 reduces, but does not completely eliminate, glycosylation by each catalytic subunit. Analogs of NGI-1 were found to have varying effects on OST inhibition corresponding to discrete chemical modifications of the small molecule. We also identified a novel compound that has specificity for the STT3B catalytic subunit. Together these findings indicate that NGI-1 alters OST fidelity and that the degree of OST activity can be pharmacologically regulated with small molecule

inhibitors. Partial inhibition of glycosylation by NGI-1 has limited effects on cell proliferation, however, one exception is tumor cells dependent on receptor tyrosine kinase glycoproteins like the epidermal growth factor receptor. Using nanoparticle delivery of NGI-1 to treat mice bearing human tumor xenografts, we demonstrate that NGI-1 significantly delays growth of tumors that are resistant to standard cancer therapies. In conclusion our data suggests that small molecules may be used to edit glycosylation of target proteins with some selectivity, providing a rationale for therapeutic development.

### (14) Anti-Inflammatory Functions of Siglec-E and Siglec-9 and Alteration of Their Ligands in Mouse Airway Inflammation and Lung Emphysema

Zi Chen[1,5], Fengrui Zhang[1], Haiying Tang[1], Ryan N. Porell[2], Ronald L. Schnaar[2], Tadahiro Kumagai[3], Michael Tiemeyer[3], Bruce S. Bochner[4], Linfu Zhou[5], Tao Zheng[1] and Zhou Zhu[1]

*[1]Yale University School of Medicine; [2]Johns Hopkins University; [3]CCRC, University of Georgia; [4]Northwestern University; [5]Nanjing Medical University*

Siglec-E in mice and Siglec-9 in humans are orthologous glycan ligand binding, regulatory molecules mainly expressed on the surface of neutrophils and monocytes. Siglec-E plays an important role in lung biology in mice. However, the functions of Siglec-E and Siglec-9 in inflammatory lung diseases, such as airway inflammation, emphysema and COPD, are not well understood. We generated transgenic mice that express human Siglec-9 and investigated its potential role in lung inflammation and emphysema, comparing with those of Siglec-E. Siglec-9 expressing transgenic mice were generated using a BAC construct containing human Siglec-9 gene components. Western blot and flow cytometry demonstrated that Siglec-9 expressing cells were predominantly Gr-1$^+$ cells and some CD11b$^+$ cells, but not other cells, as seen in humans. After back-crossing to BL/6 Siglec-E KO genetic background, we obtained Siglec-9 Tg/Siglec-E KO mice (simply Siglec-9 Tg), which were tested in LPS (5 μg/mouse, i.t. 24 hr) induced airway inflammation and neutrophil elastase (0.8U/mouse, i.t., 28 d) induced emphysema models and compared them to wild type (WT) and Siglec-E KO mice. Siglec-E and Siglec-9 specific glycan ligands were determined in lung tissues using Siglec-E-Fc and Siglec-9-Fc as probes. Bronchoalveolar lavage and lung histology revealed that WT mice had an inflammatory response in the airway and emphysematous changes in the lung tissue. Compared to WT mice, Siglec-E KO mice had significantly enhanced airway inflammation and more severe emphysema with increased chord length. Citrullinated Histone H3, a marker for neutrophil extracellular traps, was markedly increased in the lung tissue of Siglec-E KO mice as evidenced by Western blot and lung immunofluorescence. In contrast, Siglec-9 Tg mice showed inflammation and emphysema responses similar to those of WT mice, suggesting a restoration of Siglec-E function by Siglec-9 in the mouse lung. Analyses of ligand expression by overlaying Siglec-E-Fc and Siglec-9-Fc on lung tissue sections showed that in WT mouse lung, Siglec-E ligands and Siglec-9 ligands were present in airway and lung epithelial cells at baseline (PBS control) but were significantly increased in response to elastase. In Siglec-E KO lung, Siglec-E ligands and Siglec-9 ligands were strikingly increased at baseline without further increase upon elastase stimulation. In Siglec-9 Tg mouse lung, the patterns of ligand expression were more similar to those in the Siglec-E KO mouse lung. These studies revealed that Siglec-E is essential in regulating lung inflammation and emphysema, Siglec-9 largely substitutes for and restores the anti-inflammatory functions of Siglec-E *in vivo*, and Siglec-E and Siglec-9 glycan ligands are altered in lung inflammation and emphysema. The interactions between Siglec-E and Siglec-9 and their ligands in inflammatory lung diseases provide opportunities to understand and modulate these diseases. (Supported by P01HL107151)

### (15) Glycans and their binding proteins in ocular surface disease

Pablo Argueso

*Department of Ophthalmology, Harvard Medical School, Boston*

Epithelial cells lining mucosal surfaces, such as those of the ocular surface, respiratory, gastrointestinal and reproductive tracts, perform functions ascribed to maintaining a robust barrier that protects against pathogenic and environmental challenges. Integral to these functions is the carbohydrate-rich glycocalyx present along apical membranes on epithelial cells. A major component of the epithelial glycocalyx is a group of highly glycosylated, high-molecular-weight glycoproteins termed transmembrane mucins. Due to their extremely large size, they extend above other components of the plasma membrane, therefore constituting the outermost interface between the epithelial cell and the external environment. At the ocular surface, disruption of glycocalyx barrier function is associated with a wide range of disorders that include dry eye, an age-related disease affecting millions of people worldwide and whose pharmacological treatment remains unresolved. Our current studies involve investigating the interactions between transmembrane mucins and multivalent carbohydrate-binding proteins in the ocular surface epithelial glycocalyx. This field of investigation is yielding clues to the understanding of the pathogenesis of ocular surface diseases in which the glycocalyx barrier is compromised.

### (16) Regulatory functions of heparan sulfate in prostate stem/progenitor cell activities and prostatic tumorigenesis

Sumit Rai[1], Xuanyang Li[1], Houjian Cai[2] and Lianchun Wang[1]

*[1]Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA; [2]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602, USA*

Prostate cancer (PCa) is one of the most prevalent forms of malignancy and the second most common cause of cancer-related death in men. Uncovering novel mechanisms that control prostatic tumorigenesis may advance development of more effective therapeutics to treat this life-threatening disease. Heparan sulfate (HS), a type of polysaccharide, is an essential component of the cell microenvironment and plays important roles in cell-cell and cell-matrix interaction and signaling. Recent studies revealed that expressions of HS-synthesizing and modifying genes are dysregulated in human PCa, but it remains unknown what causes the aberrant HS expression, and, more importantly, what are the functional consequences of the aberrant HS expression in prostatic tumorigenesis. In this study, we explored the regulatory functions of HS in prostate stem/progenitor cell (PrSC) activities, which are responsible for adult prostate tissue homeostasis and regeneration, and in prostatic tumorigenesis. In the *in vitro* prostate sphere formation and *in vivo* prostate regeneration assays, we observed that HS is required to sustain self-renewal activity of adult PrSCs by inhibiting TGFβ signaling and functions both *in cis* and *in trans* to maintain prostate homeostasis and to fulfill prostate regeneration. In testing with the prostate-specific *Pten* knockout mice, the spontaneous prostate cancer mouse model, we observed that ablation of HS biosynthetic gene *Ext1* in the *Pten* model blocks PCa initiation and progression coupled with blockage of protumorigenic inflammatory cell infiltration, showing that HS is required to transmit the causal role of *Pten*-loss in prostatic tumorigenesis and to generate a protumorigenic microenvironment. Ongoing studies are proceeding to elucidate the underlying cellular and molecular mechanisms and to explore the potential values of aberrant HS expression as a novel biomarker for PCa early diagnosis and prognosis and targeting aberrant HS expression for PCa treatment.

### (17) Mucin-type O-glycans in intestinal mucus barrier homeostasis and diseases

Kirk Bergstrom, Jianxin Fu and Lijun Xia
*Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA*

The colonic mucus layer overlying the epithelium is at the first interface between the host and microbiota. Mucin-type *O*-linked oligosaccharides (*O*-glycans), which mainly consist of core 1- and core 3-derived *O*-glycans, are primary components of the mucus layer. Impaired *O*-glycans have been observed in patients with colitis or colon cancer, but whether these changes cause these diseases is unclear. In addition, how both types of *O*-glycan maintain mucus barrier function in the colon remains to be studied. We found that *C1galt1*, which synthesizes core 1 *O*-glycans, was expressed throughout the colon, whereas *C3GnT*, which controls core 3 *O*-glycan formation, was most highly expressed in the proximal colon. Consistent with this, mice lacking intestinal core 1-derived *O*-glycans (IEC *C1galt1*$^{-/-}$) developed spontaneous colitis primarily in the distal colon, whereas mice lacking both intestinal core 1- and core 3-derived *O*-glycans (DKO) developed spontaneous colitis in both distal and proximal colon. DKO mice showed an early onset and more severe colitis than IEC *C1galt1*$^{-/-}$ mice. Antibiotic treatment restored the mucus layer and attenuated colitis in DKO mice. Mucins from DKO mice were more susceptible to proteolysis than WT mucins. These results indicate that core 1- and 3-derived *O*-glycans collectively contribute to the mucus barrier by protecting it from bacterial protease degradation.

At older ages, DKO mice exhibited spontaneous colitis-associated colon cancers. We found that the microbiota is key for driving inflammation and tumorigenesis in the absence of core 1- and core 3-derived *O*-glycans largely through activation of epithelial caspase 1-dependent inflammasomes. Loss of O-glycan-dependent mucus barrier function results in chronic hyperactivation of caspase 1 and secretion of IL1β and IL18 into the mucosa and systemically. Depleting this pathway in DKO mice ablated iNOS production and DNA damage, and resolved most of the inflammation and cancer. These data indicate caspase 1 overactivation is completely deleterious to the host by promoting severe colitis and shaping a protumorigenic environment, and that a major function of the mucus layer is to regulate its activation by the microbiota.

In conclusion, our findings indicate mucin-type *O*-glycans constitute an innate defense barrier essential for maintaining the symbiosis between microbiota and the host. Our results suggest that inhibition of key inflammatory pathways mediated by inflammasome activation (caspase 1-dependent) may reduce the incidence of carcinogenesis in patients with ulcerative colitis.

### (18) IgA Nephropathy: An autoimmune kidney disease involving the clustered O-glycans of IgA1 as autoantigens

Matthew B. Renfrow, Tyler J. Stewart, Audra A. Hargett, Stacy Hall, William J. Placzek, Bruce A. Julian and Jan Novak
*University of Alabama at Birmingham*

Immunoglobulin A (IgA) nephropathy is the most common form of primary glomerulonephritis in the world. It is a leading cause of end-stage renal disease. This chronic kidney condition has recently been identified as an autoimmune disease involving IgA1 with altered *O*-glycosylation as the main autoantigen and target of autoantibodies. While the disease is extremely rare in central Africa, IgAN is highly prevalent in East Asia; accounting for 30–40% of the 10,000 native kidney biopsies performed annually in Japan. There has been an increasing realization that IgAN patients represent a large segment of ESRD patients worldwide, as there is no disease-specific treatment. Moreover, IgAN recurs in about 50% of IgAN patients after kidney transplantation. A better understanding of pathogenesis of IgAN offers a unique opportunity to intervene in a disease process wherein the kidneys are "innocent bystanders" that fall victim to a series of molecular events in the immune system. Included in these molecular events is a shift in the overall *O*-glycan heterogeneity of IgA1 between patients and healthy controls. The task of defining site-specific glycoprotein heterogeneity is recognized as an

area that still needs a considerable amount of effort to fully understand the role of glycan heterogeneity in biological processes and disease pathogenesis. In IgA1, we have developed quantitative ELISA and mass spectrometry assays that allow us to distinguish between IgAN patients and healthy and disease controls. Our findings have provided a means of detecting shifts in glycan heterogeneity through the application of relative quantitative, label-free analysis of the entire population of IgA1 glycoforms in a single serum sample. Our workflow allows unbiased profiling of all *O*-glycoforms in the three distinct molecular forms of IgA1 [monomeric, polymeric, and IgA1 in circulating immune complexes (CICs)] found in the serum of patients with IgAN. Additionally, we have investigated the synthesis of IgA1 *O*-glycans with a combination of glycosyltransferases on a variety of synthetic templates to understand the how clustered *O*-glycosylation on tandem repeats leads to a complex and yet restricted heterogeneity. Combined, we are developing analytical and bioinformatics tools, that allow us to understand how different IgA1 heterogeneity patterns can occur at the molecular level, including some patterns that contribute to the presentation of an autoantigen in IgA nephropathy.

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### (19) A semantic approach to Molecular Glycophenotype classification for disease diagnostics

Jean-Philippe F. Gourdine[1,3], Matthew H. Brush[1], Thomas O. Metz[2,3], David M. Koeller[1,3] and Melissa A. Haendel[1,3]
[1]*Oregon Health and Science University;* [2]*Pacific Northwest National Laboratory;* [3]*Undiagnosed Diseases Network*

Rare diseases affect 10% of the US population, and 80% of rare diseases are caused by genetic mutation. The Human Phenotype Ontology (HPO) describes enables computational description of clinical phenotypic abnormalities using a hierarchical classification of phenotype terms, including unique identifiers, synonyms, definition. HPO is the de facto standard for rare disease phenotyping for exome analysis and mechanism discovery. As part of the Monarch Initiative, HPO-based phenotype analysis tools enable the diagnosis of unknown diseases by comparing sets of phenotypes and genes associated with them. Partnerships with organizations such as the Undiagnosed Disease Program have resulted in new disease diagnoses using basic research data from model organisms and Monarch tools. Hundreds of the mutations have been found in genes involved in glycobiology either involved in glycan synthesis or degradation, or in interaction with glycan associated proteins (e.g. glycan binding protein). While glycan related diseases are abundant, to date these have been poorly described in HPO at the molecular level. For instance, specific urinary free oligosaccharides (FOS) are markers for many diseases (e.g. high mannose FOS in α-mannosidosis). These molecular glycophenotypes are well documented but are not utilized effectively in diagnostic applications. To address the lack of glycophenotype representation in the HPO, we built a molecular glyco-phenotype ontology (MGPO) that classifies glycan-related phenotypes along four essential dimensions: 1) affected glycan characteristic; 2) affected glycan type, based on the class of glycan exhibiting abnormal characteristics; 3) affected glycosylation target, based on the aglycone target affected by a defect; and 4) affected locus of abnormality, based on the subcellular location of the defect and/or the tissue or fluid. We plan to use MGPO with semantic similarity and pattern matching at the glycan level with glycomics data in the Undiagnosed Disease Network cases for diagnosis and disease/gene candidate prioritization.

### (20) ABC cassette transporters regulate glycosphingolipid biosynthesis

Monique Budani, Mustafa Kamani Budani and Clifford Lingwood
*University of Toronto, Research Institute, Hospital for Sick Children, Toronto, Canada*

The precursor for the synthesis of most complex glycosphingolipids (GSLs) is glucosylceramide (GlcCer) made on the outer Golgi membrane. The mechanism by which GlcCer is flipped into the Golgi has remained a mystery for >40 years. Based on the use of MDR1(ABCB1) inhibitors, we proposed a role for this multidrug resistance protein. We have now used a newly synthesized photolabile GlcCer crosslinker to identify GlcCer binding proteins in microsomes, using proteomics/mass spectrometry. Three ATP binding ABC cassette transporters were thus identified, ABCB10, ABCB4 and ABCA3. Using SiRNA knockdown, these candidate GlcCer flippases were reduced in DU145 cells, both in terms of total GSLs and metabolic labeling. The loss of ABC cassette proteins had a differential effect on selective GSLs. Some were increased e.g. lactosyl ceramide, while others reduced MDR1 siRNA knockdown reduced overall GSL content by >50% according to cell line. These studies provide new insights into the complex regulation of GSL biosynthesis by precursor supply, a new basis for the link between cancer/multidrug resistance and GSL biosynthesis and indicate that multiple ABC transporters can transport GlcCer into the Golgi to generate LacCer pools used for the synthesis of different GSLs. Different LacCer pools could provide a partial basis for the biosynthesis of different GSL series within different Golgi regions

### (21) Transport of lipopolysaccharides across the bacterial cell envelope

Natividad Ruiz
*Department of Microbiology, The Ohio State University*

The cell surface of most Gram-negative bacteria is covered with LPS, a glycolipid that creates a permeability barrier against many small, hydrophobic toxic molecules. As a result, these bacteria are naturally resistant to many antibiotics and

detergents. In addition, LPS is essential for viability in some bacteria and can be a potent inducer of the immune system. In order to assemble this glycolipid on their cell surface, bacteria must extract newly synthesized LPS from the inner membrane, and then transport it across the aqueous periplasm and through the outer membrane. This transport of LPS from the inner membrane to the cell surface is facilitated by seven Lpt (LPS transport) proteins, LptA-G, which are thought to assemble into a trans-envelope complex. At the inner membrane, the Lpt system includes an ATP-binding cassette (ABC) transporter composed of the cytoplasmic ATPase LptB and the transmembrane domains LptFG. $LptB_2FG$ performs the unusual task of extracting LPS from the outer leaflet of the inner membrane and loading it into the LptCAD periplasmic bridge. To begin to understand how this ABC transporter functions, we are characterizing the $LptB_2FG$ system in *Escherichia coli* using genetic and biochemical approaches. In these studies, we have identified novel domains in $LptB_2FG$ that are essential for LPS transport. These domains are involved in substrate recognition and in coordinating ATPase activity with LPS transport. We hope that this knowledge will help in the discovery of small molecules that inhibit Lpt and thereby compromise the permeability barrier function of the outer membrane, rendering Gram-negative pathogens sensitive to antibiotics.

### (22) Identifying lipid scramblases for dolichol-based glycolipids involved in protein *N*-glycosylation in the endoplasmic reticulum

Anant K. Menon
*Department of Biochemistry, Weill Cornell Medical College*

Dolichol-based glycolipids are key components of protein glycosylation pathways in the endoplasmic reticulum (ER), responsible for contributing the monosaccharides and oligosaccharides necessary to construct *N*-glycoproteins, *O*- and *C*-mannosylated proteins, and GPI-anchored proteins in the lumen of the ER. Because they are synthesized on the cytoplasmic face of the ER, these glycolipids must be flipped across the ER membrane to participate in luminal glycosylation reactions. The protein transporters (scramblases) that catalyze the transbilayer transport of these lipids have not been identified at the molecular level although their activity has been characterized using ER microsomes as well as proteoliposomes reconstituted with rat liver and yeast ER membrane proteins. New approaches will be presented that are directed toward the discovery of the scramblases that transport $Mannose_5GlcNAc_2$-PP-dolichol and Glucose-P-dolichol across the ER membrane.

### (23) A New Hypothesis For Lec5

Mark A. Lehrman and Hua Lu
*UT Southwestern Medical Center*

In the late 1970s/early 1980s, CHO-K1-derived lipid-linked oligosaccharide (LLO) mutants of the Lec5 genotype were identified. The essential properties of Lec5 (B211) cells, reported by Sharon Krag's group using radioisotopic labeling methods, were (a) robust deficiency of Glc-P-Dol dependent LLO glucosylation, with milder loss of Man-P-Dol dependent LLO mannosylation, (b) dependence of LLO phenotype upon low cell density, (c) temperature sensitivity, and (d) isolation of ts revertants which were also reverted for the LLO phenotype. A deficiency of dolichol-P metabolism and thus Glc-P-Dol production was suggested. However, the Lec5 gene has never been identified, and the precise mechanistic basis for the Lec5 phenotype was not determined.

We reexamined authentic Lec5 cells (obtained from Pamela Stanley) with steady-state non-radioactive methods, by fluorophore-assisted carbohydrate electrophoresis. All of Krag's essential observations (a-d) were replicated. In addition low-density Lec5 mutants accumulated free oligosaccharides (fOS), but in contrast to the LLO phenotype, fOS accumulation was not corrected in ts/LLO revertants. A Man-P-Dol dependent non-LLO lipid-associated glycan, most likely a GPI, also accumulated in Lec5 mutants. However, Man-P-Dol and Glc-P-Dol appeared normal in low-density Lec5 mutants.

These new observations suggest an alternative hypotheses for Lec5 in which sugar-P-dolichol donors, lipid acceptors, and corresponding transferases are all present, but the donors are not utilized. Such a phenotype is reminiscent of Lec35 mutant cells with deletions in the MPDU1 (mannose-P-dolichol utilization) gene, which fail to use both Man-P-Dol and Glc-P-Dol. Like Lec5, Lec35 mutants also accumulate fOS. In 2001 we reported that the cDNA we had cloned for MPDU1 was unexpectedly highly selective for Man-P-Dol. This implicated an alternative transcript encoded by the same gene, with specificity for Glc-P-Dol, but the hypothesized transcript was not identified.

With hindsight, it is possible that Lec5 and Lec35 represent weak and strong alleles, respectively, of the same gene, with Lec5 having selective loss of a theoretical Glc-P-Dol specific MPDU1 isoform. Its high reversion rate suggests that Lec5, unlike Lec35, may be controlled epigenetically.

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### (24) LmeA, a Periplasmic Membrane-Bound Protein, is Critical for Lipomannan Biosynthesis and Cell Envelope Integrity in Mycobacteria

Yasu S. Morita, Sarah Osman and Kathryn C. Rahlwes
*Department of Microbiology, University of Massachusetts, Amherst, MA, 01003, USA*

The integrity of the multilaminate glycan-rich cell envelope surrounding mycobacteria is critical to its survival and pathogenesis. The prevalence of phosphatidylinositol mannosides, lipomannan (LM) and lipoarabinomannan (LAM) in the cell envelope suggests their important roles in the mycobacterial life cycle. Recent studies identified lectin receptors for these mycobacterial glycolipids, which are expressed on the surface of mammalian immune cells, indicating their importance in the host-pathogen interactions. However, these glycolipids are

also important for the integrity of the mycobacterial cell envelope. Indeed, deletion of the *pimE* gene (Δ*pimE*) encoding the first committed step in phosphatidylinositol hexamannoside biosynthesis in *Mycobacterium smegmatis* results in the formation of smaller colonies than wildtype colonies on Middlebrook 7H10 agar. Taking advantage of the colony morphology defect, we isolated spontaneous suppressor mutants of Δ*pimE* that reverted to wild-type colony size. Out of 22 suppressor mutants, six mutants accumulated significantly smaller LM and/or LAM. Genome sequencing revealed mutations in genes involved in the LM/LAM biosynthesis such as the arabinosyltransferase EmbC and the mannosyltransferase MptA. Furthermore, we identified three suppressor mutants that carried different mutations in a previously uncharacterized gene, *MSMEG_5785*, that we designated *lmeA* for lipomannan elongation factor A. Complementation of these suppressor mutants with the *lmeA* gene restored the original Δ*pimE* phenotypes, and deletion of *lmeA* in wild-type *M. smegmatis* (Δ*lmeA*) resulted in smaller lipomannan as seen in the suppressor mutant. LmeA carries a predicted N-terminal signal peptide, and density gradient fractionation and detergent extractability experiments indicated that LmeA localizes to the cell envelope. Using a lipid ELISA assay, LmeA was found to bind to plasma membrane phospholipids such as phosphatidylethanolamine and phosphatidylinositol. LmeA is widespread throughout the *Corynebacteriales* order and predicted to be an essential gene in *Mycobacterium tuberculosis*. These data indicated that LmeA is an evolutionarily conserved cell envelope protein critical for controlling the mannan chain length of LM/LAM. Our preliminary data further suggest that Δ*lmeA* is more sensitive to antibiotics than the wild-type, implying that the cell envelope integrity is significantly compromised in this mutant. Currently, we are investigating the potential defect of the Δ*lmeA* cell envelope and the essentiality of the gene in *M. tuberculosis*.

### (25) Making home sweet and sturdy: Investigations into the roles of glycosylation in the cyst wall of Toxoplasma gondii

T. Tomita[1], T. Sugi[1], R. Yakubu[1], V. Tu[1], Y. Ma[1], Louis Weiss[1,2,*]

*Albert Einstein College of Medicine, Departments of [1]Pathology and [2]Medicine, Bronx, New York, 10461*

*louis.weiss@einstein.yu.edu*

Toxoplasma gondii, an Apicomplexan parasite, can cause significant morbidity in both immune competent and immunocompromised humans presenting at encephalitis and/or chorioretinitis. Within its host disease is a consequence of the transition of bradyzoites, found within tissue cysts in neurons into actively replicating tachyzoites. These tissue cysts are cloaked by a glycosylated structure termed the cyst wall, which is critical for survival, reactivation and transmission of this pathogen. We identified the glycoprotein CST1 which localizes to the cyst wall, confers structural rigidity to cysts, and acts as a critical scaffold for other cyst wall proteins (CWPs). The mucin-like domain of CST1 is composed of 20 units of threonine-rich tandem repeats that are O-GalNAc glycosylated and is critical for the functions of CST1. In other eukaryotes a family of enzymes termed polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts) initiates O-GalNAc glycosylation. To identify which isoforms of ppGalNAc-Ts are responsible for the glycosylation of the CST1 mucin-like domain and to evaluate the function of each ppGalNAc-T in the overall glycosylation of the cyst wall, all five ppGalNAc-T isoforms were deleted individually from the T. gondii genome. The ppGalNAc-T2 and -T3 deletion mutants produced various cyst wall glycosylation defects. Both T2 and T3 glycosylate the CST1 mucin-like domain, and this glycosylation is necessary for CST1 to confer structural rigidity on the cyst wall. Furthermore, we found that T2 is required for the initial glycosylation of the mucin-like domain and T3 is responsible for the sequential glycosylation on neighboring acceptor sites, demonstrating hierarchical glycosylation by two distinct initiating and filling-in ppGalNAc-Ts in an intact organism. A longstanding limitation of parasite glycoprotein analysis is that purification of parasites from their host cells results in the elimination secreted proteins including CWPs and host translocated proteins (HTPs). We have adapted a simple and robust α-cyclopropyl ester (αCP)-porcine liver esterase (PLE) paired bioorthogonal chemistry system to selectively label parasite glycoproteins while minimizing the labeling of host glycoproteins. This system allows the labeling and subsequent purification of these CWPs and HTPs facilitating their recovery. This system has enabled us to better characterize glycoproteins in the matrix and cyst wall of T. gondii. This αCP-PLE system can be used to also examine other sugars (e.g. GlcNAc, ManNAc, Fucose), as well as to assess the direct effect of various inhibitors on intracellular parasites. These various studies that allow the delineation of the biological function(s), composition, and interactions of CWPs will drive the development of new approaches for the control of latency in T. gondii. [Supported by NIH grants R21AI123495 and R01AI095094]

### (26) Elucidating glycomic contributions to Toxoplasmabiology and virulence

Christopher M. West[1,2], Travis Ichikawa[1,2], Elisabet Gas-Pascual[1,2], Osman Sheikh[1,3], Mariam I. Serji[1,3], Msano Mandalasi[1,2], Rick Tarleton[2] and Lance Wells[1,3]

*[1]Dept. of Biochemistry & Molecular Biology, University of Georgia, Athens, GA; [2]Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA; [3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA*

Protozoan parasites employ glycosylation pathways to mediate protein and cellular functions in ways that both parallel and diverge from those in other kingdoms of life. Owing to its complexities, roles of glycosylation in cellular regulation are often overlooked. To address this we embarked on a systematic CRISPR/Cas9 gene editing strategy to connect

glycogenes with the glycome and cellular functions. Sequence and motif searches were used to compile candidate genes for glycan assembly for two important intracellular human pathogens, *Toxoplasma gondii* (this presentation) and *Trypanosoma cruzi*. Guide RNAs for single and double CRISPR editing were selected using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) and incorporated into a plasmid designed for transient expression with Cas9 into most strains of *Toxoplasma*. Edited clones were selected by repair-mediated integration of a DHFR amplicon encoding resistance to pyrimethamine, followed by PCR confirmation. Whole cell permethylated glycomes were profiled using direct infusion Orbitrap MS-MS and queried using the GRITS database. Targeting of N-glycosylation genes confirmed reliance on parasite rather than host genes for precursor assembly and revealed unanticipated complexity. Targeting O-glycosylation confirmed parasite assembly of the Glc-Fuc-disaccharide on thrombospondin repeats. Targeting of the parasite homolog of the nucleo-cytoplasmic O-GlcNAc-transferase indicated it utilizes GDP-Fuc rather than UDP-GlcNAc. Targeting of other predicted cytoplasmic glycosyltransferases showed roles in assembling a hydroxyproline-dependent O-glycan on an E3-ubiquitin ligase. Several of the edited strains exhibited slow growth on fibroblasts, which involves cycles of invasion, proliferation and egress consistent with potential roles in virulence. These and all other vectors and strains in our pipeline are available to the community. Supported by NIH grants R21-AI123161 (Common Fund) and R01-GM084383.

### (27) Neoglycoproteins as biomarkers for cutaneous leishmaniasis

Alba L. Montoya[1], Krishanthi Subramaniam[4], Alvaro Acosta-Serrano[4], Igor C. Almeida[2,3] and Katja Michael[1,3]
*[1]Department of Chemistry, University of Texas at El Paso; [2]Department of Biological Sciences, University of Texas at El Paso; [3]Border Biomedical Research Center, University of Texas at El Paso; [4]Department of Parasitology and Department of Vector Biology, Liverpool School of Tropical Medicine*

Leishmaniasis is an infectious vector-borne disease caused by a number of different species of the protozoan parasite *Leishmania*. There are different forms of leishmaniasis, the most common being cutaneous leishmaniasis (CL), which is associated with large, open skin ulcers that may heal over several months, but can lead to complications due to secondary infections. CL affects ~ 12 million people worldwide, particularly in Northern Africa, the Middle East, Asia, and in Latin America. CL is also on the rise in the U.S. mostly due to military deployment to endemic regions (e.g., Afghanistan and Iraq). The presentation of CL can be confused with other skin conditions, such as leprosy or skin cancer, and proper diagnostic tools (Polymerase Chain Reaction) are often not available in developing countries. Thus, there is a need for the development of other robust and reliable diagnostic tools for the accurate diagnosis of CL. *Leishmania* spp. express unusual glycans on their cell surfaces that are foreign to humans, which may elicit a strong antibody (IgG) response in patients. Specifically, it is known that the glycoinositol phospholipids (GIPLs) of *L. major* (old world) and *L. mexicana* (new world) contain unusual alpha-galactopyranosyl (alpha-Gal*p*) and beta-galactofuranosyl (beta-Gal*f*)-residues, which suggests that these *Leishmania* species are serologically detectable and also distinguishable from other *Leishmania* species that do not express these glycans. Here we present the interrogation of a synthetic glycoarray consisting of alpha-Gal*p* and beta-Gal*f*-containing neoglycoproteins with sera of CL patients from Saudi Arabia using chemiluminescent Enzyme-Linked Immunosorbent Assay technology. We demonstrate that the sera from CL patients who have active lesions due to infections with *L. major* show antibody reactivity against certain synthetic alpha-Gal*p* and/or beta-Gal*f* epitopes when compared to a heterologous control group and to a group of cured patients. Our data suggest that certain glycotopes show promise as biomarkers for the diagnosis of CL by *L. major* infection and for follow-up after chemotherapy.

### (28) Identifying the in vitro Arginine-GlcNAcylation targets of the NleB/SseK family of effectors

Nichollas E. Scott, Josh Newson, Georgina L. Pollock, Jaclyn S. Pearson and Elizabeth L. Hartland
*Department of Microbiology and Immunology, University of Melbourne at the Peter Doherty Institute for infection and Immunity, Victoria, Australia*

Effector glycosyltransferases are a growing class of secreted bacterial proteins which subvert normal cellular functions through the modification of host proteins with carbohydrates. Recently a novel family of effector glycosyltransferases were identified within the enteric pathogens (enteropathogenic *Escherichia coli (EPEC), Salmonella enterica* serovar Typhimurium and *Citrobacter rodentium)* which mediate the addition of N-acetylglucosamine to arginine residues. The archetype of this family, NleB1 of EPEC, has been shown to modify a range of death-domain-containing proteins including FADD, TRADD and RIPK1. Yet, these targets were identified under ectopic expression with the true targets during infection still unclear. To define the substrates Arginine-glycosylation during *in vitro* we have developed an Arginine-GlcNAcylation affinity proteomic approach. Utilizing this approach, we have characterized the repertoire of Arginine-GlcNAcylation during NleB1 ectopic expression, EPEC infections under different level of NleB1 expression and *C. rodentium* infections. We show that multiple host targets can be Arginine-GlcNAcylated when non-authentic levels of NleB1 are introduced. Yet, during EPEC and *C. rodentium* infections Arginine-117 of FADD is rapidly and preferentially subjected to modification. Temporal profiling of Arginine-GlcNAcylation showed that during EPEC infections alternative Arginine-GlcNAcylation substrates only appeared after FADD modification or in response to overexpression of NleB1. Similarly, homologues of

NleB1 such as SseK1 and SseK3 from *Salmonella enterica* serovar Typhimurium also demonstrate a restricted repertoire during infection compared with overexpression. Interestingly within Salmonella infections, both SseK1 and 3 target different subset of death-domain-containing proteins within the host which are unique to those targeted by NleB1. These findings demonstrate that the NleB/SSeK effectors can act far more promiscuously than previously thought when overexpressed but under endogenous infections conditions are highly specific

**(29) Active Roles for Heparan Sulfonated Proteoglycans and Growth Factors in Human Papillomavirus Infectious Entry: A Trojan Horse Mechanism?**

Michelle A. Ozbun

*The Maralyn S. Budke Endowed Professor of Viral Oncology, The University of New Mexico School of Medicine and Comprehensive Cancer Center, Albuquerque, NM 87131 USA*

Human papillomavirus (HPV) infections are restricted to squamous epithelial cells and can occur in both mucosal and cutaneous epithelia. Primarily, HPVs cause benign warts or papillomas, but a subset of HPV infections is causally related to the development of a variety of human cancers. Like a number of pathogens, HPV entry into target host cells (human keratinocytes) is initiated by first binding to heparan sulfonated proteoglycan (HSPG) moieties present at the cell surface and on the extracellular matrix (ECM)/basement membrane. The virus is thought then to move to distinct secondary receptors, which are responsible for particle internalization. Despite intensive investigation, the mechanism of HPV movement to and the nature of the secondary receptors have been unclear. We find that a large fraction of HPV16 particles binds to the ECM *via* heparan sulfate (HS) chains, and that the HSPG syndecan-1 (snd-1) molecules present in the ECM are involved in virus binding. Inhibiting the normal processing of snd-1 and HS molecules *via* matrix metalloproteinases (MMPs) and heparanase dramatically reduces virus release from the ECM, cellular uptake and infection. Conversely, exogenous heparinase activates each of these processes. We confirm that HPV16 released from the ECM is infectious in keratinocytes. We show that HPV16 particles are not liberated from bound HSPG attachment factors by dissociation, but rather reside in infectious soluble high molecular weight complexes with HSPG, including syndecan-1 and bioactive compounds, like growth factors. Employing a co-culture assay, we demonstrate HPV associated with soluble HSPG-growth factor complexes can infect cells lacking HSPG. Interaction of HPV-HSPG-growth factor complexes with growth factor receptors leads to rapid activation of signaling pathways important for infection, whereas a variety of growth factor receptor inhibitors impede virus-induced signaling and infection. Depletion of syndecan-1 or epidermal growth factor and removal of serum factors reduce infection, while replenishment of growth factors restores infection. Our findings support an infection model whereby HPV usurps normal host mechanisms for presenting growth factors to cells *via* soluble HSPG and HS complexes as a novel "Trojan Horse" method for interacting with entry receptors independent of direct virus-cell receptor interactions. Portions of this work have been published in PLoS Path. 2012, 8:e1002519; J. Gen. Virol. 2015, 96:2232.

**(30) Changes in cell surface glycans in women with bacterial vaginosis and impact on Fusobacterium vaginal colonization**

Kavita Agarwal[2], Lloyd S. Robinson[2], Lynne Foster[2], Hueylie Lin[2], Nicole M. Gilbert[3], Warren G. Lewis[1] and Amanda L. Lewis[2,3]

*[1]Department of Medicine, Washington University in St. Louis; [2]Department of Molecular Microbiology, Washington University in St. Louis; [3]Department of Obstetrics and Gynecology, Washington University in St. Louis*

Bacterial vaginosis (BV) is an important risk factor associated with adverse health outcomes such as acquisition of secondary infections and pre-term birth. BV is characterized by a dysbiosis of the vaginal microbiota, in which *Lactobacillus* levels are low and diverse anaerobes overgrow. Women with BV are more likely to be vaginally colonized with *Fusobacterium nucleatum*, a pathogen frequently isolated from amniotic fluid in cases of pre-term birth. However, mechanisms of *Fusobacterium* vaginal colonization have not been studied. Microbial sialidase activity in vaginal fluids is a diagnostic feature of BV and is known to be produced by BV-associated bacteria such as *Gardnerella* and *Prevotella*. Interestingly, *F. nucleatum* does not produce sialidase of its own, but often colonizes mucosal surfaces that are home to sialidase-producing bacteria (e.g. gut, mouth and vagina).

Here we show at least two mechanisms by which sialidase-producing bacteria in BV can promote the colonization or overgrowth of secondary pathogens such as *F. nucleatum*. First, we show that free sialic acids are also available at higher concentrations in BV-positive women. Through bioinformatic analysis, we found that genes required for metabolic utilization of free sialic acid are present in multiple subspecies of *F. nucleatum*. Using *in-vitro* experiments we show that *F. nucleatum* encodes as functional sialic acid lyase (*nanA*) and can uptake and metabolize free siaclic acid released from sialylated substrates by *G. vaginalis* and *Prevotella* sialidases. In addition to this, we also generated a mutation in the sialic acid transporter (*siaT*) in *F. nucleatum* 23726 and show that it is more rapidly cleared than its wild-type counterpart from the vaginas of mice with sialidase-positive, but not sialidase-negative vaginal microbiotas. These studies suggest that free sialic acids made available by sialidases of the endogenous microbiota are metabolized by *F. nucleatum* and facilitate more persistent vaginal colonization.

Second, our studies revealed that women with BV exhibit a pronounced depletion of sialic acids from vaginal epithelial cells and exposure of sugar residues such as galactose (Gal) and *N*-acetyl-galactosamine (GalNAc). We also provide evidence that *F. nucleatum* adheres to exposed carbohydrate

binding sites containing Gal and GalNAc residues on mammalian cell surfaces. Monosaccharide analogs like *O*-benzyl-GalNAc, that mimic GalNAc-α-Ser/Thr (Tn Antigen), can be used to inhibit such interactions *in-vitro*. Specific galactoside-containing oligosaccharides in human milk were also effective inhibitors of *F. nucleatum* adhesion to de-sialylated glycans.

Taken together, these data provide the first mechanistic glimpse of how sialidase activity, one of the biochemical hallmarks of BV, may help to create a more hospitable environment for potential reproductive tract pathogens.

### (31) Antibody fucosylation restricts Fc gamma receptor IIIA (CD16A) N-glycan motion to reduce affinity

Adam W. Barb, Daniel J. Falconer and Ganesh P. Subedi
*Roy J Carver Dept of Biochem, Biophys & Mol Biol, Iowa State University, Ames*

Therapeutic monoclonal antibodies (mAbs) are largely based on the immunoglobulin G1 (IgG1) scaffold and many elicit a cytotoxic cell-mediated response by binding Fc gamma receptors. Core fucosylation, a prevalent modification to the asparagine(N)-linked carbohydrate on the IgG1 crystallizable fragment (Fc), reduces Fc gamma receptor IIIA (CD16A) binding affinity and mAb efficacy. We determined IgG1 Fc fucosylation reduced CD16A binding by $1.7 \pm 0.1$ kcal/mol when compared to afucosylated IgG1 Fc, however, CD16A N-glycan truncation decreased this penalty by 70%. Fc fucosylation restricted the manifold of conformations sampled by the CD16A N162-glycan with the greatest effect on the linkage between [alpha]mannose(1–6)[beta]mannose residues that samples space proximal to the IgG1 Fc fucose residue. Fucose also slowed ms-ms motions of the N297-glycan as observed by solution NMR and perturbed IgG1 Fc structure and this motion may account for the remaining 30% penalty of fucosylation. Our results indicated the CD16A N162-glycan modulates antibody binding indirectly through conformational sampling, as opposed to a direct mechanism that contributes stabilizing contacts as is observed for lectin/ligand interactions. An indirect mechanism is rapidly tunable in situ by modifying N-glycan composition and doesn't require changes at the gene or polypeptide level to modulate affinity.

### (32) Sialic acid structures and linkages - variation within animals and effects on virus interactions

Colin Parrish[1], Brian Wasik[1], Karen Barnard[1] and Ajit Varki[2]
[1]*Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University;* [2]*Glycobiology Research and Training Center, University of California, San Diego*

Many pathogens use Sialic acids (Sia) as primary receptors for cell entry, and they often express proteins that bind to Sias, modify them, or cleave them off. Sias are found on cells on glycans attached to glycolipids or glycoproteins, and they are also present in the mucus that protects mucosal surfaces. Sias may be modified in a number of ways (9-O-, 7,9-O-, 4-O-acetyl, 5-N-glycolyl among others), and are attached to underlying glycans through different linkages – and those vary between different hosts and tissues. While the role of Sia linkages has been explored as controlling the tropisms of some pathogens, the effects of most modified Sias are often not well understood. However, some O-acetyl-modified Sias have been identified as influenza infection inhibitors (horse or guinea pig serum – likely Neu4,5Ac) or as likely inhibitors of NA efficiency (Neu5,9Ac), while in the cases of influenza C and D Neu5,9Ac are the primary receptors. Here I will review the background to the interactions between Sia and pathogens (particularly viruses), discuss the detection of the modified Sia in tissues, and also describe some examples of the variant expression in the tissues of different animals. A summary of what is known about the effects of those modified Sia on pathogens will be provided, along with an outline of how new molecular tests and tools provide an opportunity to more clearly understanding the effects of Sia expression and modifications on pathogen interactions with cells and tissues, as well as on host tropisms.

### (33) Inhibition of O-glycan biosynthesis using hexosamine analogs

Sriram Neelamegham[1], Shuen-Shiuan Wang[1], Virginia del Solar Fernandez[1], Gino Stolfa[1], Aristotelis Antonopoulos[2], Anne Dell[2], Stuart M. Haslam[2], Mehrab Nasirikenari[3], Joseph T. Lau[3] and S. G. Sampathkumar[4]
[1]*Department of Chemical and Biological Engineering, State University of New York, Buffalo, NY, USA;* [2]*Department of Life Sciences, Imperial College London, London, UK;* [3]*Department of Cellular and Molecular Biology, Roswell Park Cancer Institute, Buffalo, NY, USA;* [4]*Laboratory of Chemical Glycobiology, National Institute of Immunology, New Delhi, India*

Robust metabolic strategies to inhibit O-linked glycosylation are currently lacking. We tested the efficacy of a panel of N-acetylgalactosamine (GalNAc) analogs to reduce cellular O-glycosylation biosynthesis and leukocyte adhesion to the selectin family of adhesion molecules. Among the compounds tested, GalNTGc, a C-2 sulfhydryl substituted GalNAc was metabolically active. The culture of HL-60 promyelocytic leukocytes with this hexosamine analog reduced cell-surface sialyl Lewis-X expression by 50–80%. Such treatment also reduced cell adhesion to L- and P-selectin bearing substrates by 50-75% ex vivo in microfluidics based flow chamber studies, and it reduced P-selectin dependent HL-60 binding to activated platelets under hydrodynamic shear. In mechanistic studies, GalNTGc dramatically increased the binding of VVA-lectin to HL-60s by > 10-fold, indicating the truncation of O-glycan biosynthesis. Consistent with this, the molecular mass of mucinous proteins, PSGL-1 and CD43, was also reduced by 20–25%. Mass spectrometry based glycome profiling confirm profound inhibition of O-glycan elaboration upon treatment with GalNTGc, with negligible change in N-linked glycosylation. Studies with Maleimide-FITC labeling of GalNTGc

treated cells suggests low levels of direct GalNTGc incorporation into cellular glycoconjugates, with this carbohydrate and its derivatives being noted on cellular O-glycans, N-glycans, and carbohydrates attached to glycosphingolipids. When bone marrow cells from donor animals were cultured with GalNTGc for 2-days prior to infusion into recipients, GalNTGc was observed to reduce granulocyte migration to sites of inflammation in a murine peritonitis model. Directly feeding GalNTGc to mice over 4-days prior to the induction of peritonitis also reduced granulocyte homing to sites of inflammation by ~50%. Thus, GalNTGc is a pharmacologically active compound that may reduce selectin mediated leukocyte adhesion in vivo. More significantly, it is a robust and specific O-linked glycosylation inhibitor that could be widely useful for a range of applications.

### (34) New insights into glycoconjugate receptors for cholera toxin

Jakob Cervin[2], Amberlyn M. Wands[1], Anirudh Sethi[1], He Huang[3], Ye Zhang[3], Nicole S. Sampson[3], Jennifer J. Kohler[1] and Ulf Yrlid[2]

*[1]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, United States; [2]Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden; [3]Department of Chemistry, Stony Brook University, Stony Brook, New York, United States*

Cholera toxin (CT) is a bacterial toxin that enters and intoxicates mammalian host intestinal epithelia cells, resulting in a massive loss of fluids and electrolytes that causes the severe dehydration that characterizes the disease cholera. To initiate this intoxication process, the B subunit of CT (CTB) first binds to cell surface receptors displayed on the apical surface of the intestinal epithelia. While the monosialoganglioside GM1 has been widely accepted to be the sole receptor for CT, we recently discovered that intestinal epithelial cell lines also utilize fucosylated glycan epitopes on glycoproteins to facilitate cell surface binding and endocytic uptake of the toxin. We will report on ongoing work aimed at (1) more completely characterizing the identity of fucosylated CT receptors, (2) evaluating the role of GM1 and GM1-related glycosphingolipids for CT action in vivo, and (3) using competitive interference with CTB binding to fucosylated receptors as a strategy to block CT action. Our findings open up new avenues to block CT intoxication and to design detoxified enterotoxin-based adjuvants.

### (35) Unique features of the machinery that pathogenic and commensal microbes use to attack host glycans

Alisdair Boraston

*Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, V8W 3P6, Canada*

*Streptococcus pneumoniae* is a bacterium that normally asymptomatically and transiently inhabits the human nasopharynx. This bacterium, however, can readily change its role into that of a deadly pathogen, causing a wide array of invasive and lethal infections and it is thus a leading cause worldwide of morbidity and mortality from an infectious disease. As antibiotic resistance is a critical problem in the clinical profile of *S. pneumoniae*, this pathogen was recently listed by the World Health Organisation as one of the top 12 priority "bacteria for which new antibiotics are urgently needed." Efforts to understand the relationship between this bacterium and its human host have led to an emerging theme that strongly links *S. pneumoniae*'s capacity to colonize and cause invasive disease with its ability to process host carbohydrates, making these metabolic systems potential therapeutic targets. In this presentation I will discuss the key carbohydrate metabolizing pathways that contribute to the virulence of the bacterium with a particular focus on a newly reported pathway that targets high-mannose N-glycan degradation.

### (36) Who will win? The endless battle between bacteria and their viruses in the gut

Christine M. Szymanski[1,2], Jessica C. Sacher[2], Clay S. Crippen[1], Robert T. Patry[1], Yan-Jiun Lee[3], Martin Stahl[4], Michael Rothrock[5], Susan Sanchez[6], Bruce A. Vallance[4] and Peter R. Weigele[3]

*[1]Department of Microbiology and Complex Carbohydrate Research Center, University of Georgia, Athens; [2]Department of Biological Sciences, University of Alberta, Edmonton; [3]New England BioLabs, Ipswich, Massachusetts; [4]Division of Gastroenterology, BC Children's Hospital, The Child and Family Research Institute, University of British Columbia, Vancouver; [5]Agricultural Research Service, United States Department of Agriculture, Athens; [6]College of Veterinary Medicine, University of Georgia, Athens*

We often consider microbial adaptation results from interactions with larger mammalian hosts or with the smaller"animalcules" that Leeuwenhoek observed in his ancient microscope. However, most bacteria face even greater threats from their microbial counterparts and predatory bacteriophages that evolve in similar time frames and consequently engage them in a constant arms race. Bacteria are comprised of the same 4 macromolecules that constitute all living matter: carbohydrates, proteins, lipids and nucleic acids. The latter 3 classes are often further modified with a diverse array of monosaccharides leading many researchers to conclude that all biological interactions involve sugars. We have tracked bacteria such as the ubiquitous foodborne pathogen, *Campylobacter jejuni*, that is normally a commensal in the chicken gut, immunologically silent and therefore not threatened by its host. Nonetheless, *C. jejuni* is heavily armed with the ability to vary its surface glycoconjugates through a mechanism of DNA slipped-strand mispairing. This sloppy mismatch repair system creates a pool of *C. jejuni* single strain variants and affords select members of the population the ability to thrive under varying stressors. These pressures could be due to immune responses activated during

accidental entry into the human host, diverse environmental conditions the organism must navigate through, or overwhelming phage numbers *C. jejuni* encounters within the intestines of its hosts.

We identified a novel O-methyl phosphoramidate (MeOPN) modification, not previously described in nature, that commonly modifies *C. jejuni* capsular polysaccharides (CPS) of varying serotypes. The unusual phosphorus-nitrogen bond is believed to predate the emergence of life and is created by the phosphorylation of glutamine through a *C. jejuni* specific glutamine kinase. Many phages target the MeOPN moiety for *C. jejuni* recognition and infection. To counteract phage attack, *C. jejuni* encodes phase-variable MeOPN transferases to prevent CPS attachment of the phage receptor. The organism also encodes phase-variable Me transferases that can be used to obstruct this receptor by decorating adjacent sugar residues. Through these mechanims of CPS variation, *C. jejuni* can not only survive phage attack in chicken ceca, but within a matter of days, the organism is isolated in the same numbers as from birds not phage-exposed. *C. jejuni* also assembles lipopolysaccharides (LOS) that mimic the ganglioside structures abundant in the intestinal brush borders of its avian host. Phage-encoded AB5 toxins, such as cholera toxin, specifically bind to the *C. jejuni* LOS and prevent its growth. To counteract this growth inhibition, *C. jejuni* varies its LOS sugar structures averting toxin recognition. Although it appears that our microbe is winning the battle, we have also identified mechanisms that promote phage predation of *C. jejuni* and other microbes in the avian gut. The presentation will summarize these findings.

### (37) Understanding Influenza A Specificity: An Evolution of Paradigms

Ye Ji, Yohanna J.B. White, Oliver C. Grant and Robert J. Woods
*Complex Carbohydrate Research Center, University of Georgia*

Understanding the molecular origin of influenza specificity is complicated by the paucity of quantitative affinity measurements, and the qualitative and variable nature of glycan array data. Further obstacles arise from the varied impact of viral glycosylation on specificity, and the relatively narrow spectrum of biologically-relevant receptors present on glycan arrays. A survey of receptor conformational properties leads to the novel conclusion that conformational entropy differences play a key role in defining specificity, as does the newly-reported [1] ability of biantennary receptors that terminate in Siaα2-6 sequences to form bidentate interactions (two branches in a single receptor binding simultaneously to two binding sites in a hemagglutinin trimer).

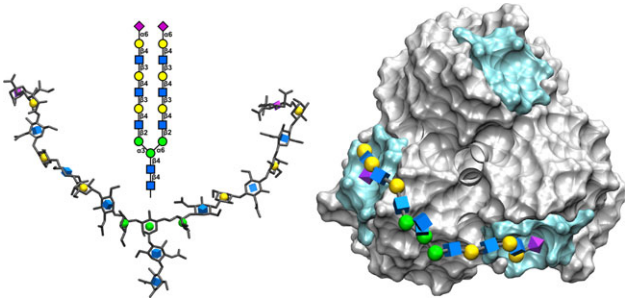

**Figure 1.** Proposed [1] bidentate binding of a biantennary α2-6 glycan (3D-SNFG representation [2]) to the HA (grey surface) from a pandemic H1N1 (A/California/04/2009).

Bidentate binding provides a functional explanation of the observation that Siaα2-6 receptors adopt an open-umbrella topology when bound to hemagglutinins from human-infective viruses, and calls for a reassessment of virus avidity and tissue tropism.

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### (38) Nascent microbiome and early metabolism are perturbed by pre- and post-natal exposure to artificial sweeteners

Stephanie Olivier-Van Stichelen, Kristina I. Rother and John A. Hanover
*National Institute of Health, NIDDK*

Artificial sweetener (AS) consumption is increasing globally, mostly as an attempt to reduce sugar consumption and total caloric intake. Currently, the average American ingests ~22 teaspoons of added sugar every day. This modern glucose-rich diet correlates with increases in the prevalence of obesity, diabetes and others metabolic syndromes. While substitution of sugars with artificial sweeteners such as aspartame, sucralose, acesulfame-potassium and saccharin may appear healthier for metabolic homeostasis than a diet rich in carbohydrates, recent studies have shown that AS also impair glucose metabolism notably through gut microbiota alterations. This has presented questions about the excessive use of these food additives. Furthermore, little is known about pre- and post-natal exposure to AS and their effect on microbiota establishment. In this study, we exposed mice to a AS containing diet (acesulfame-potassium + sucralose) and observed the effect on microbiome colonization and early metabolism in the offspring. We observed that AS exposure, presumably though placental circulation and breast milk, dramatically altered the gut microbiome of 19 day old pups. The largest change was the disappearance of one major bacterial group, the mucin-feeding bacteria *Akkermansia muciniphila*. This taxon constitutes approximately 35% of the microbiome of control 19 day old pups. Interestingly, *A. muciniphila*'s absence is associated with obesity, diabetes and inflammation; its presence is usually linked to a healthy metabolism. In

addition, we also noticed an increase in firmicutes in AS fed pups, which has been linked to metabolic deregulation as well. Interestingly, the mothers were only slightly affected by AS exposure suggesting that pups are more sensitive to AS diet through placental circulation and breastfeeding. The pups also demonstrated low weight and decreased fasting glucose associated with key changes in amino acids, lipids and carbohydrate metabolism, suggesting metabolic deregulation early in life. Most of the affected metabolites were found in the gut more than plasma, confirming that AS affect directly gut metabolic balance. The prenatal exposure to AS might alter the gut environment (notably mucins), changing colonization of this major component of the gut microbiome. Thus, pre- and post-natal AS diet dramatically alters microbiome transmission/establishment and more precisely the colonization of the mucin-feeding *A. muciniphila*. This early alteration of the microbiome impacts early metabolic homeostasis and this perturbation may persist into adulthood. This study raises concerns the widespread AS consumption during pregnancy and lactation.

### (39) Plant based glycan engineering for the production of therapeutic proteins

Herta Steinkellner
*University of Natural Resources and Life Sciences, Vienna*

Plants are increasingly being recognized for the production of recombinant therapeutic proteins and recently it was shown that plants are highly amendable to glycan engineering approaches. Paradoxically, the limited endogenous glycosylation repertoire has turned out to be an advantage for generating homogeneous glycans, in contrast to the large glycome and the resulting glycan heterogeneity in CHO cells that impedes the targeted manipulations. We have developed a plant based expression platform enabling extensive engineering of glycans towards complex targeted structures. The system is based on the use of a glyco-engineered *Nicotiana benthamiana line* lacking plant specific glycosylation, instead synthesizes core human-type complex N-glycans, i.e. GnGn structures (doi: 10.1111/j.1467-7652.2008.00330.x.). This structure, common in all higher eukaryotes, serves as acceptor for further N-glycan diversifications. The plant based approach uses a modular switch of glyco-engineered *N. benthamiana* mutants in combination with transient expression modules harboring mammalian glycosylation enzymes. Using this approach we were able to produce proteins with a varietly of predefined glycosylation profiles, up to a complexity level of polysialylated structures (>40 sialic acid residues) (doi: 10.1073/pnas.1604371113.). Such unusual polymer formation with its strong negative charge not only changes a protein's functionality but may also be used *per se* in therapeutic applications (e.g. in inflammatory settings). Harvesting of recombinant proteins one week post DNA construct delivery allows high speed and flexibility (doi: 10.1016/j.copbio.2014.06.008.). This presentation provides an overview of the current front in plant based glycan engineering processes, bottlenecks and challenges, which a special focus on generation of different antibody formats (IgG, IgA, IgE and IgM).

### (40) Rapid mapping of glycoprotein structure-activity relationships by shotgun scanning glycomutagenesis

Matt DeLisa
*Cornell University*

It remains a significant challenge to predict *a priori* what sites in a protein can/will become glycosylated and what effect glycan installation at a particular site will have on protein structure and function. Shotgun scanning mutagenesis combines the concepts of alanine scanning mutagenesis and binomial mutagenesis with phage display technology, allowing an extremely rapid method for analyzing the effect of many side chains simultaneously without the need for protein purification or biophysical analysis of each individual mutant. At present, while the presence/absence of glycans can be assessed by a one-site-at-a-time approach, *there is no analogous shotgun scanning technique for rapid, high-throughput determination of many glycan sites in a single experiment*. To address this technology gap, we have developed an experimental procedure termed shotgun scanning glycomutagenesis (SSGM) for comprehensively identifying the sites/structural motifs within a target protein that can/cannot tolerate glycan installation. SSGM uses combinatorial protein libraries in which glycosylation "sequon walking" is used to introduce an acceptor site at every possible position along the protein backbone, although the multi-residue nature of a sequon, *e.g.*, N-X-S/T or D/E-X1-N-X2-S/T, necessitates insertion of two or four additional amino acid substitutions at each position. The library of sequon variants is interrogated for the presence/absence of glycosylation using a high-throughput screen, such as our previously described glycoSNAP (glycosylation of secreted *N*-linked acceptor proteins) technology. As proof-of-concept, we used SSGM to investigate the permissiveness to glycosylation of the Im7 immunity protein, a 90-residue, four-helix bundle protein that has been extensively studied including with respect to limited site-specific glycosylation. Following library construction and screening, a surprisingly large number of sites along Im7 were found to be glycosylated, with efficiencies ranging from 30–100%. Because our screen distinguishes positive and negative clones, we are able to tabulate an exhaustive map of the sites in the target protein that can tolerate glycan installation as well as those that cannot. We anticipate that SSGM will become a useful new tool for deep exploration of glycoprotein structure-activity relationships (glycoSARS) as well as for developing glycoengineered protein variants with new or improved functions.

### (41) Developing Anti-inflammatory Drugs Targeting Selectins

Richard D. Cummings[1], Richard Alvarez[2] and Rodger P. McEver[3]

*[1]Department of Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215; [2]Tetherex Pharmaceutical Corporation, Oklahoma City, OK 73104; [3]Cardiovascular Biology Research Program, Oklahoma City, OK 73104*

Selectins are key adhesion molecules regulating leukocyte trafficking in homing to lymph nodes and in the process of inflammation. While selectins recognize sugar ligands, such recognition is often necessary but not sufficient for high affinity and specific cellular interactions. In this regard, we and others have studied such molecular interactions and means of regulating and blocking these interactions. Insights into this area could have enormous health benefits especially in treating chronic inflammatory diseases. Two of the major drug targets in this are P-selectin and its glycoprotein ligand PSGL-1. In attempting to block interactions of P-selectin and PSGL-1, we developed multiple approaches, including blocking antibodies and synthetic glycosulfopeptides (GSPs). Based on multiple patents in this area, in 2002 we founded an Oklahoma City-based biotechnology company that was named Selexys Pharmaceuticals Corporation. That company was successful in developing a humanized anti-P-selectin antibody to block P-selectin functions in humans. In a phase 2 clinical trial, the anti-P-selectin antibody reduced vaso-occlusive pain crises in patients with sickle cell disease, leading to an acquisition of the company by Novartis in 2016. This presentation will deal with the history of the founding and development of the company, as an example of the challenges of translational glycobiology and drug development in this area. Such developments of specific drugs targeting pathways involving carbohydrates and their recognition, and their success in treating human diseases, bode well for future growth of glycobiology into all aspects of medicine.

### (42) Identification and Characterization of Multiple Missense Mutations in the O-GlcNAc transferase gene that are causal for X-linked Intellectual Disability

Nithya Selvan[1], Krithika Vaidyanathan[1], Stephan George[1], Tejasvi Niranjan[2], Chin Fen Teo[1], Melanie May[3], Sneha Patel[1], Brent Weatherly[1], John Opitz[4], John Carey[4], David Viskochil[4], Jozef Gecz[5], Fatema J. Serajee[6], AHM M. Huq[6], Yunhui Peng[7], Emil Alexov[7], Tao Wang[2], Charles Schwartz[3] and Lance Wells[1]

*[1]Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA.; [2]McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD 21287, USA.; [3]Greenwood Genetic Center, Greenwood SC 29646, USA.; [4]Pediatrics (Medical Genetics), Pediatric Pathology, Human Genetics, Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah.; [5]Department of Paediatrics and Robinson Research Institute, The University of Adelaide, Adelaide, South Australia, 5006, Australia.; [6]Department of Pediatrics and Neurology, Wayne State University, Detroit, MI 48201, USA.; [7]Department of Computational Biophysics and Bioinformatics, Clemson University, Clemson, SC 29634, USA*

X-linked intellectual disability (XLID) affects ~1 in 1,000 males. While there a multitude of known causal genes a significant portion of XLID is of unknown genetic etiology. Here we demonstrate that a variety of different missense mutations in O-GlcNAc transferase (OGT) segregate with XLID in multiple families. OGT is the sole glycosyltransferase responsible for the regulatory O-GlcNAc modification of hundreds of nuclear and cytosolic proteins and is an essential gene. The majority of the mutations result in amino acid replacements in the tetratricopeptide (TPR) repeats and not the catalytic domain of OGT. Here we present the clinical phenotypes of the affected individuals, the sequencing data of the families including skewed X-inactivation in carrier females, and the mutations discovered to date. We go on to biochemically characterize the XLID recombinant variants of the enzyme including stability, dimerization, O-GlcNAc protein/peptide kinetics, and HCF-1 protease activity. We also document a compensation mechanism for maintaining O-GlcNAc levels present in patient lymphoblasts for one of the mutations where OGT regulates the transcription of OGA. We also will present our preliminary data using Cas9-engineered human embryonic stem cells that carry the various mutations described including impact on steady-state levels of OGT, O-GlcNAcase (OGA), and global O-GlcNAc levels as well as on global transcription via RNA-Seq. Thus, more than 30 years after the discovery of nucleocytoplasmic O-GlcNAc by the Hart laboratory, mutations in the coding region of one of the genes for a cycling enzyme, OGT, have been directly linked to a human disease, XLID.

### (43) Aberrant glycosylation in breast cancer results in modulation of the immune microenvironment

Richard Beatson, Virginia Tajadura-Ortega, Gianfranco Picco, Thomas Noll, Paul Crocker, Joyce Taylor-Papadimitriou and Joy Burchell

*Kings College London*

Cancers have developed a plethora of mechanisms to evade the immune response including initiating a permissive local environment. Indeed for cancer cells to remodel their immune micro-environment, they need to acquire changes that include the recruitment and 'education' of monocytes and the repolarization of resident macrophages.

We have shown that the interaction of a tumour-associated glycoform of MUC1 expressed by breast carcinomas with the lectin Siglec-9 found on monocytes and macrophages, can act as such an immune microenvironment remodeling trigger. Siglec-9 is a member of the Siglec family of sialic acid binding

lectins expressed by immune cell subsets and this family is involved in controlling the immune response to self and has recently been shown to be involved in tumour immune evasion.

We have shown that when a defined cancer-specific glycoform of MUC1 (MUC1-ST) engages Siglec-9 on monocytes, the monocytes are induced to release factors associated with immune recruitment, immune modulation and tumour growth. Moreover, when MUC1-ST binds to Siglec-9 expressed by macrophages these myeloid cells are induced to undergo phenotypic change to become tumour-associated macrophages expressing high levels of CD206, CD163, IDO and importantly PDL-1; a phenotype associated with a poor prognosis for breast cancer patients.

This work defines a critical role for aberrantly glycosylated MUC1 and suggests a new potential therapeutic approach by developing novel innate glycan checkpoint inhibitors, such as Siglec-9 blocking antibodies.

### (44) The consequences of human ganglioside deficiency

Michael Tiemeyer[1], Michelle Dookwah[1], Charles Schwartz[2], Kevin A. Strauss[3], Heather Flanagan-Steet[1], Richard Steet[1] and Kazuhiro Aoki[1]

*[1]CCRC, University of Georgia; [2]Greenwood Genetic Center; [3]Clinic for Special Children*

Human diseases that result from altered glycosphingolipid catabolism are well known, including disorders such as Tay-Sachs and GM1 gangliosidosis. These lysosomal storage diseases greatly impact normal neural function, although the mechanistic connection between lysosomal accumulation and disease progression remains poorly understood. More recently, human disorders of glycosphingolipid biosynthesis have been identified, providing an opportunity to assess the impact of glycosphingolipid deficiency. One of these disorders results in loss-of-function of ST3Gal5 (GM3 synthase) with the concomitant loss of all complex gangliosides. This disorder, known as Salt & Pepper Syndrome in a cohort of African-American patients and as Old Amish Epilepsy Syndrome in the Amish community, is associated with devastating neurodevelopmental, neurosensory, and seizure phenotypes. To understand the underlying pathophysiology, we have undertaken the development of induced pluripotent cell lines (iPSCs) from affected patients and derived neuronal populations from these iPSCs. These derived cell lines have allowed us to investigate cell-type specific glycomic alterations and to assess the impact of altered ganglioside profiles on cell signaling, cell survival, and cellular homeostasis relevant to the human disease.

### (45) A Family of Carbohydrate Tumor Antigens with a Proposed Common Mechanism of Action

Marit Sletmoen[1], Thomas A. Gerken[2], Bjorn T. Stokke[3], Joy Burchell[4] and Fred Brewer[5]

*[1]Department of Biotechnology and Food Science, The Norwegian University of Science and Technology, NO-7491 Trondheim, Norway; [2]W. A. Bernbaum Center for Cystic Fibrosis Research, Departments of Pediatrics and Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4948, USA; [3]Biophysics and Medical Technology, Department of Physics, The Norwegian University of Science and Technology, NO-7491 Trondheim, Norway; [4]Breast Cancer Biology, King's College London, Guy's Hospital, London, SE1 9RT, UK; [5]Departments of Molecular Pharmacology, and Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

The human glycome is altered in many diseases including cancer. For example, the mucin-type O-glycome aberrantly expresses the truncated glycans Tn (GalNAca1-Ser/Thr) and STn (Neu5Aca2,6GalNAca1Ser/Thr) in over 80% of carcinomas. Indeed, Tn and STn have been tumor markers for colorectal, lung, breast, cervical and gastric carcinomas for over three decades, and the expression level of Tn correlates with the metastatic potential and poor prognosis of patients. However, the role of Tn and STn in cancer and other diseases is not well understood. Our recent discovery of the self-binding properties (carbohydrate-carbohydrate interactions) of Tn (Tn – Tn) and STn (STn – STn) (1), and the binding and cross-linking activities of these two cancer markers provides a model for their possible roles in cellular transformation. We also present evidence for a family of carbohydrate tumor antigens and blood group determinants that include Tn and STn, which also appear to possess carbohydrate-carbohydrate interactions and participate in oncogenesis.

**Reference**

1. Haugstad KE, Hadjialirezaei S, Stokke BT, Brewer CF, Gerken TA, Burchell J, Picco G, Sletmoen M. 2016. Interactions of mucins with the Tn or Sialyl Tn cancer antigens including MUC1 are due to GalNAc-GalNAc interactions. *Glycobiology*, 26:1338–1350.

### (46) Fut2 is required for methacholine-induced airway hyperreactivity in a mouse model of allergic asthma

Dorota S. Raclawska, Rachel Waagmeester, Adrianne L. Stefanski and Christopher M. Evans

*Department of Medicine, University of Colorado Denver, Aurora, CO*

The polymeric mucins Muc5ac and Muc5b are the predominant glycoproteins in airway mucus. Muc5b is present at baseline and is crucial for host defense. Muc5ac is the major polymeric mucin up-regulated in allergic mouse lungs. We recently showed that Muc5ac is required for airway hyperreactivity (AHR) to the bronchoconstricting agent methacholine (MCh) through its central role in acute mucus plugging. Based upon these findings, we postulate that structural characteristics such as glycosylation dictate distinct functions of Muc5ac and Muc5b in health and disease. We

hypothesize that in an allergic model of asthma, the potential for Muc5ac to mediate mucus plugging and AHR is determined by α1,2-linked fucosylated glycans on Muc5ac. To test this, *Fut2* gene deficient mice and WT controls were challenged with an allergen, *Aspergillus oryzae* extract (AOE), via aerosol (10% v/v) weekly 4 times. Forty-eight hours after the last challenge, mice were analyzed for mucin production, inflammation, and lung function. Presently, Muc5ac and Muc5b glycan structures are poorly understood, so *Muc5ac* and *Muc5b* deficient mice were also used to assess the specificity of mucin fucosylation. We found that both Muc5ac and Muc5b are heavily α1,2-fucosylated and that α1,2-fucosylation is specifically facilitated by Fut2. In pulmonary function studies measured using a flexiVent, Fut2 was required for AHR to MCh. Preliminary histologic examination of lungs from AOE-challenged *Fut2* knockout mice and WT controls suggest that *Fut2* knockout mice have reduced mucus plugging, and we are currently using a stereological point counting technique to quantitate and confirm this observation. Eosinophilic inflammation was not significantly different between WT control (n = 9) and Fut2 knockout (n = 10) animals, further implicating reduced mucus plugging as the chief mechanism for protection from AHR in Fut2 deficient mice. In human asthma, mucus hypersecretion is an important cause of morbidity and mortality, and *FUT2* expression by mucin-producing cells is associated with increased risk of hospitalization due to asthma exacerbations. Based on these findings, we propose that targeting glycosylation on mucins could be an effective therapeutic approach in allergic asthma.

Keywords: mucin, Fut2, allergic asthma, hyperreactivity

### (47) Mapping sites and molecular functions of O-glycosylation

Katrine T. Schjoldager, Shenjun Wang, Hiren J. Joshi, Yang Mao and Sergey Y. Vakhrushev
*Copenhagen Center for Glycomics, Department of Molecular Medicine, University of Copenhagen*

The worldwide implementation of gene editing technologies has massively advanced the fields of glycosciences and biotechnology and with knockout and knockin of glycogenes we have developed e.g. isogenic cell models for disorders of glycosylation. We have used genetic engineering and state-of-the-art mass spectrometry to characterize O-GalNAc glycoproteomes of human cell lines and address the roles of disease associated polypeptide GalNAc-transferases (GalNAc-Ts). Isogenic cells with and without individual members of the large GalNAc-T family have enabled identification of non-redundant contributions of individual GalNAc-T isoforms to the O-glycoproteome and evaluation of biological functions conferred. In particular, we have confirmed that the *GALNT2* gene is a modulator of plasma lipids *in vitro* and *in vivo* as predicted from several genome-wide association studies. More recently we have established how another isoform *GALNT11* directs isoform-specific glycosylation on low-density lipoprotein (LDL) related receptors and we are now interrogating the potential function of O-GalNAc glycans in the LDL class A repeats in this large class of receptors. Broader aims of these efforts are to use genetic engineering of model cells to provide cell-based production of well-defined glycoproteins and cellular glycophenotypes to explore biological functions of glycans and biomedical applications.

### (48) Essential roles of O-GlcNAcylation in B cell activation

Kuo-I Lin[1], Jung-Lin Wu[1], Pan-Hung Hsu[2], Yu-Ju Chen[3] and Takashi Angata[4]
[1]*Genomics Research Center, Academia Sinica, Taipei 115, Taiwan;* [2]*Department of Life Science and Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 202, Taiwan;* [3]*Institute of Chemistry, Academia Sinica, Taipei 115, Taiwan;* [4]*Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan*

*O*-linked β-N-acetylglucosamine (*O*-GlcNAc) modification (*O*-GlcNAcylation) is a post-translational modification that adds a GlcNAc to serine or threonine residue of nuclear and cytosolic proteins. *O*-GlcNAcylation is catalyzed by *O*-GlcNAc transferase (OGT) and is removed by *O*-GlcNAcase (OGA). *O*-GlcNAcylation regulates many aspects of proteins, including protein stability, localization and activities, and plays important roles in many diseases such as diabetes, neurodegenerative diseases and cancer. The functional role and molecular mode of action of protein *O*-GlcNAcylation in B cells is not well understood. B cell activation is triggered by the engagement of B cell receptor (BCR) with antigen, thereafter inducing the differentiation of antibody-secreting plasma cells or memory B cells. We showed that protein *O*-GlcNAcylation accumulates after cross-linking of BCR by anti-IgM in mouse splenic B cells and that inhibition of OGA by a specific inhibitor, thiamet G, promotes anti-IgM-mediated activation of B cells. Comparative phosphoproteomic analyses reveled several *O*-GlcNAc-dependent phosphoproteins in B cell activation. We further created a mouse line in which *Ogt* is deleted in a B cell-specific manner to demonstrate that *Ogt* ablation impairs BCR cross-linking induced cell activation and survival. By comparative *O*-GlcNAc proteome analysis of wild type and *Ogt* knockout splenic B cells using sWGA, a lectin that has high affinity for *O*-GlcNAc, we identified a key BCR signaling molecule modified by *O*-GlcNAc and demonstrated that its activity is regulated by *O*-GlcNAcylation. These results demonstrate that B cells rely on *O*-GlcNAc to efficiently induce BCR signaling.

### (49) Drosophila N-glycanase 1 (Pngl) regulates BMP autoregulation in the Drosophila intestine

Antonio Galeone[1], Seung-Yeop Han[1], Chengcheng Huang[2], Akira Hosomi[2], Tadashi Suzuki[2] and Hamed Jafar-Nejad[1]
[1]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, United States;* [2]*Glycometabolome Team, RIKEN Global Research Cluster, Saitama, Japan*

In *Drosophila melanogaster, Pngl* encodes a deglycosylation enzyme that acts on *N*-glycoproteins and exhibits a high degree of functional conservation with human NGLY1 (*N*-glycanase 1). Mutations in human *NGLY1* cause a rare congenital disorder with severe developmental delay, delayed bone age and osteopenia, gastrointestinal dysfunction, small hands/feet and absent tears. However, the mechanism by which NGLY1 and its homologs regulate animal development and the pathophysiology of *NGLY1* deficiency in human patients are not known. Here, we report that *Pngl* mutants show developmental midgut defects reminiscent of midgut-specific loss of BMP signaling. Tissue-specific knockdown and rescue experiments indicate that *Pngl* is primarily required in the mesoderm during *Drosophila* development. The enzymatic activity of Pngl is essential for BMP autoregulation in the visceral mesoderm mediated by Dpp as ligand and Tkv as receptor. Genetic and phenotypic analyses indicate that loss of BMP signaling in *Pngl* mutant midguts results from a requirement for Dpp homodimers in this tissue, suggesting that the heterodimer form of Dpp with Gbb, the other fly BMP ligand, is not affected. Indeed, biochemical data show that loss of *Pngl* results in a severe decrease in the level of Dpp homodimers, suggesting a role of Pngl for homodimer formation and/or stability. I will discuss our ongoing experiments to elucidate how the *N*-glycanase activity of *Pngl* regulates BMP signaling. Given the high level of conservation that exists between fly and mammalian BMP ligands, NGLY1 might play a similar role in BMP processing and dimerization in mammals as well.

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### (50) Identification of Siglec-15 ligands using proximity labeling method

Yi-Hsiu Chen[1], Penk-Yeir Low[1], Yi-Ju Chen[2], Yu-Ju Chen[2] and Takashi Angata[1,3]

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

Siglecs are a family of vertebrate lectins that recognize sialic acids and participate in self-nonself discrimination by the immune system. Identification of Siglec ligands is technically challenging, because the affinity between Siglecs and their ligands tends to be low. To overcome this problem, we developed a method to identify Siglec ligands by proximity labeling. In brief, the cells expressing Siglec ligands are first labeled with FLAG-tagged recombinant Siglec protein coupled with peroxidase-conjugated anti-FLAG antibody (Siglec-peroxidase complex). The cells are then incubated with hydrogen peroxide and biotin-tyramide, which is converted to a short-lived radical and covalently ligate to the protein in the vicinity of the Siglec-peroxidase complex. Biotinylated proteins are affinity-purified and identified by mass spectrometry. As a proof of concept, we used recombinant CD22/Siglec-2 to label its ligands on human B-lymphoma cell line, and identified several glycoproteins including CD45, IgM, and endogenous CD22 itself, which have beenpreviously reported to be CD22 ligands.

Siglec-15 is involved in osteoclast differentiation, but its ligand has not been identified. We applied the proximity labeling method to identify Siglec-15 ligands on RAW264.7 mouse macrophage cell line, which is often used as an osteoclast precursor model. We identified dozens of Siglec-15 ligand candidates by the proximity labeling method, and further demonstrated that some of these ligand candidates physically associates with Siglec-15 on RAW264.7 cells. Knock-down of one of these ligand candidates by shRNA, as well as that of Siglec-15, resulted in the reduced RAW264.7 cell fusion induced by RANKL. Taken together, our results demonstrated that the proximity labeling method is useful for the identification of functionally relevant Siglec ligands.

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### (51) LNFPIII-Dex conjugates function in vivo to normalize metabolic function in High-Fat Diet Obese mice

Donald Harn[1], Jessica Ramadhin[1], Richard Meagher[5], Suresh Ambati[5], Nikolay Filipov[2], Lisa Shollenberger[1] and Thomas Norberg[4]

*[1]Department of Infectious Diseases, University of Georgia; [2]Dept. if Physiology And Pharmacy, University of Georgia; [3]College of Family and Consumer Sciences, University of Georgia; [4]Dept. of Chemistry, Uppsala University, Uppsala Sweden; [5]Dept. of Genetics, University of Georgia*

Obesity is epidemic in the United States with approximately 32% of the adult population defined as obese. Obesity elevates inflammation in the body increasing the risk for fatty liver disease, Type 2 Diabetes, microbiome dysbiosis, leaky gut syndrome and cardiovascular diseases. In an earlier study we demonstrated that systemic treatment of High-Fat Diet/Diet Induced Obesity (HFD/DIO) obese mice with LNFPIII-Dex conjugates, was sufficient to reverse fatty liver disease and restore insulin sensitivity. We have expanded these studies to examine sex-specific differences in metabolic homeostasis in obese mice and in response to LNFPIII-Dex conjugates. LNFPIII-Dex is a conjugate composed of 10–12 molecules of Lacto-N-fucopentaose III (LNFPIII) attached to a 40 kDa dextran carrier. LNFPIII is a human breastmilk sugar containing Lewis$^x$, a structure known for its immune-modulating properties. LNFPIII-Dex treatment also improves insulin signaling via upregulation of *cebpa*, *glut4*, *insrb*, and *irs2* and enhances liver function via downregulation of lipogenic genes (*fas*, *acc1/2*, *scd1*, and *srebp-1c*). Initial studies involving female Diet Induced Obese (DIO) mice treated with LNFPIII-Dex demonstrate improved glucose tolerance with an additional decrease in WAT mass. Moreover, LNFPIII-Dex treatment of female DIO mice leads to increased *glut4*, *ppara*, *pparg*, *ppargc1a*, and *ucp1* in visceral adipose tissue. Interestingly, DIO mice treated with LNFPIII-Dex had little evidence of leaky gut

syndrome, in contrast to control (Dex) treated mice. Similarly, LNFPIII-Dex treatment of DIO mice normalized the gut microbiome similar to that seen in lean, normal diet mice, whereas Dex treated DIO mice demonstrated significant microbiome dysbiosis. We next asked what happens during pregnancy as roughly 1/3 of women entering pregnancy in the U.S. are considered obese. Obesity during pregnancy is a significant risk factor for increased adverse health outcomes in children. Thus, we performed studies where we initiated LNFPIII-Dex treatment of DIO female mice beginning at conception. These experiments showed that LNFPIII-Dex treatment during pregnancy was sufficient to normalize several health/metabolic parameters in pups, whereas Dex treatment did not. Thus, LNFPIII-Dex conjugates demonstrate promise in both male and female Diet induced Obesity models via mechanisms related to inflammation, insulin signaling, lipolysis, and adipogenesis. Ongoing studies will directly compare male and female DIO mice to further determine the mechanism via which LNFPIII-Dex ameliorates obesity and metabolic disease.

### (52) Siglec-9 Recognizes Sialylated Keratan Sulfate Glycoproteins on Human Airways

Steve M. Fernandes[1], Ryan N. Porell[1], Anabel Gonzalez Gil[1], Simone Kurz[2], Kazu Aoki[2], Michael Tiemeyer[2] and Ronald L. Schnaar[1]

[1]*Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD;* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA*

Sialic acid binding Ig-like lectins (Siglecs) are immuno-regulatory molecules expressed on distinct subsets of immune cells. Siglec-9, which upon engagement inhibits inflammatory cells, is expressed on human neutrophils as well as monocytes, dendritic and NK cells. Dysregulation of Siglec-9 signaling may be involved in neutrophilic inflammatory disorders including chronic obstructive pulmonary disease (COPD), where neutrophils accumulate in the trachea, alveoli and lung parenchyma. In the current study, Siglec-9 sialoglycan ligands were extracted from post-mortem human airway tissue with 6 M guanidinium hydrochloride, separated by size-exclusion chromatography, resolved by agarose-acrylamide composite gel electrophoresis, blotted, and probed with human-Fc chimeras of Siglec-9. Size-separated ligands were further purified using a His-tagged pentameric Siglec-9 chimera bound to nickel Sepharose beads. Affinity purified Siglec-9 sialoglycan ligands were subjected to proteomic mass spectrometry, revealing MUC5B among the purified proteins that migrated at > 4 MDa and DMBT1 among the isolated proteins that migrated at 600 kDa. Based on evidence that sulfated sialoglycans may engage siglecs, we tested the purified Siglec-9 ligands for susceptibility to keratanase I & II enzymatic treatments. Keratanase I treatment resulted in loss of Siglec-9 binding to both the purified >4 MDa and 600 kDa bands, whereas equivalent keratanase II treatment did not alter Siglec-9 binding. Purified ligands were fully sensitive to sialidase enzymatic treatment but not to chondroitinase ABC or heparinase I and III. Differential keratanase I sensitivity suggested that sialylated sulfated keratan sulfate chains with relatively low sulfation levels on glycoproteins extracted from human airways bind Siglec-9. These data are consistent with glycan array and neoglycolipid ELISA data demonstrating Siglec-9 binding to 6-sulfo-sialyl LacNAc (Neu5Acα2-3Galβ1-4[6 S]GlcNac) glycans. Supported by the Lung Inflammatory Disease Program of Excellence in Glycoscience (http://lidpeg.org; P01HL107151) and NIGMS T32GM080189.

### (53) An actin-related trafficking protein modulates neural-specific glycosylation in the Drosophila embryo

Simone Kurz, Kazuhiro Aoki, Sarah Baas Robinson and Michael Tiemeyer

*CCRC, University of Georgia*

Precise protein glycosylation is essential for normal development and tissue function. However, the pathways and mechanisms controlling tissue-specific glycan expression are incompletely understood. In *Drosophila*, the distribution of a subset of N-linked glycans known as HRP epitopes is tissue restricted, providing a useful model system to identify control mechanisms driving tissue specific glycosylation. We have previously described a mutation in the *Drosophila* tollo/toll-8 gene that alters neural-specific expression of HRP epitopes. To expand our understanding of the tollo/toll-8 pathway, we performed a non-complementation screen using a set of first chromosome deletions crossed to homozygous *tollo*$^{-}$ mutants in order to identify interacting genes. Deletions that failed to complement the loss of HRP-epitopes in *tollo*$^{-}$ heterozygotes were further investigated for the presence of interacting loci. Only 1 of 44 deletion stocks, that together covered the entire first chromosome, exhibited loss of HRP-epitope in trans with a single *tollo*$^{-}$chromosome. The deleted chromosomal segment mapped to 14C5-15B1. Based on mRNA expression in neural tissues, the top candidate gene mapping to this cytological region is an actin-related trafficking protein (Arp2). Embryos carrying a disrupted Arp2 gene ($Arp2^{f04069}$) demonstrate reduced HRP-epitopes in embryos by western blot. Since HRP-epitopes account for less than 1% of the total N-linked glycans in wildtype embryos, we improved our workflow for N-glycan analysis to include LC-MS based separation and MS/MS characterization of permethylated N-glycans. Comparison of glycan profiles detected by direct infusion MS to those detected by LC-MS verified the reduction of HRP-epitopes in the $Arp2^{f04069}$ embryos. Targeted analysis of glycogene transcripts and Golgi morphology indicate that the molecular composition and architecture of the secretory pathway is altered in $Arp2^{f04069}$ embryos. We propose that disruption of Arp2 affects the regulation of actin dynamics and alters secretory pathway dynamics, resulting in a reduction of HRP-epitopes.

### (54) Development of a Rapid 2-AB Sample Preparation Workflow for N-Glycan Release and Labeling

Andres Guerrero, Vaishali Sharma, John Yan, Aled Jones, Michael Kimzey, Emily Dale, Ted Haxo and Sergey Vlasenko
*ProZyme, Hayward CA*

The characterization of N-glycans is essential to the development of biotherapeutics. Typically, enzymatically-released N-glycans are derivatized with a tag to allow for fluorescence (FLR) and mass spectrometry (MS) detection by HILIC UHPLC-FLR and UHPLC-MS. N-glycan sample preparation often requires numerous hours or days to complete. 2-AB (2-aminobenzamide) is a fluorescent tag that has been used to generate N-glycan data for more than 20 years and is well established in many laboratories. Presented herein is the development and application of a rapid N-glycan sample preparation workflow utilizing a 5-minute in solution deglycosylation step followed by direct on matrix 2-AB labeling and cleanup without the need for a dry down step, samples are ready for analysis in approximately 2 hours.

We present a comparison study consisting of common biotherapeutic molecules, including monoclonal antibodies and FC fusion proteins using three different N-glycan sample preparation workflows offered by ProZyme: GlykoPrep, Gly-X 2-AB Express and Gly-X 2-AB using with a non-sodium cyanoborohydride reductant. Gly-X 2-AB Express offers a much improved time to result compared to traditional sample preparation methods.

### (55) Effects of sialic acid biosynthesis on N-linked glycan structure, cell surface interactions, and muscle diseases of aging

Nam D. Pham[1], Poh-Choo Chang[2], Soumya Krishnamurthy[1], Amberlyn M. Wands[1], Paolo Grassi[2], Anne Dell[2], Stuart M. Haslam[2] and Jennifer J. Kohler[1]
[1]*University of Texas Southwestern Medical Center;* [2]*Imperial College London*

GNE myopathy is a rare muscle disorder associated with aging and is related to sporadic inclusion body myositis (sIBM), the most common acquired muscle disease of aging. The pathogenesis of sIBM is currently unknown and there are no effective treatments for the disease. Understanding the pathogenesis of the related disease GNE myopathy may provide new therapeutic targets for sIBM. GNE myopathy is associated with mutations in UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE). GNE harbors two enzymatic activities required for biosynthesis of sialic acid in mammalian cells. Mutations to both GNE domains are linked to GNE myopathy. However, correlation between mutation-associated reductions in sialic acid production and disease severity is imperfect. To investigate other potential effects of GNE mutations, we compared sialic acid production in cell lines expressing wild-type or mutant forms of GNE. Although we did not detect any differences attributable to disease-associated mutations, lectin binding and mass spectrometry analysis revealed that GNE deficiency is associated with unanticipated effects on the structure of cell-surface glycans. In addition to exhibiting low levels of sialylation, GNE-deficient cells produced distinct N-linked glycan structures with increased branching and extended poly-N-acetyllactosamine (polyLacNAc). GNE deficiency may affect levels of UDP-GlcNAc, a key metabolite in the nutrient-sensing hexosamine biosynthetic pathway, but this modest effect did not fully account for the change in N-linked glycan structure. Further, GNE deficiency and glucose supplementation acted independently and additively to increase N-linked glycan branching. Notably, N-linked glycans produced by GNE-deficient cells displayed enhanced binding to galectin-1, indicating that changes in GNE activity can alter affinity of cell-surface glycoproteins for the galectin lattice. These findings suggest an unanticipated mechanism by which GNE activity might affect signaling through cell-surface receptors. Moreover this work provides new insight into pathways that may be involved in muscle diseases of aging.

### (56) Dissecting the function of the O-GlcNAcase HAT-like domain using genetic, biochemical and structural biology approaches

Andrii Gorelik, Andrew Ferenbach, Olawale Raimi and Daan van Aalten
*Centre for Gene Regulation and Expression, School of Life Sciences, University of Dundee, UK*

O-GlcNAcylation is an abundant and important post-translational modification of proteins. Despite the diverse roles of this modification in protein stability, protein-protein interactions and interplay with other post-translational modifications, its cycling on serines and threonines of proteins is controlled by a single pair of enzymes: O-GlcNAc transferase (OGT) and O-GlcNAc hydrolase (OGA), both of which are essential in mammals.

O-GlcNAcase possess two domains. Glycoside hydrolase domain, located at the N-terminus, is required for the catalytic activity of OGA. On the other hand, the function of the C-terminal domain of OGA, which has a sequence similarity to GCN5 acetyltransferases but does not bind AcCoA, has remained unknown. We aimed to dissect the function of the HAT-like domain of OGA using a combination of genetic, biochemical and structural biology approaches.

First, we investigated the localisation of OGA by overexpressing wild type O-GlcNAcase and O-GlcNAcase lacking the HAT-like domain in the OGA-null background (CRISPR/Cas9 knockout) of HeLa cells. Microscopy and fractionation data indicated localisation of both proteins in the cytoplasm. We then performed a pulldown of these overexpressed proteins coupled to mass spectrometric analysis to study the interactome of the HAT-like domain. Fatty acid synthase, which was previously reported to interact with O-GlcNAcase, was enriched in the full length OGA sample. In addition, we report

a novel interaction of O-GlcNAcase with ATP-citrate synthase, another key enzyme in fatty acid metabolism.

To validate the interactions with the HAT-like domain of OGA *in vivo* and assess the activity of the truncated enzyme, we performed CRISPR/Cas9 deletion of the C-terminal domain of O-GlcNAcase. The interactions of OGA with fatty acid synthase and ATP-citrate synthase were validated by a co-immunoprecipitation experiment. Apart from mediating O-GlcNAcase interactions, we show that the HAT-like domain is important for the full catalytic activity of OGA *in vivo*. The mutant cells exhibited elevated O-GlcNAc levels, whereas OGT levels were not significantly perturbed, implying that the HAT-like domain is essential for full activity of O-GlcNAcase.

We obtained the first crystal structure of the HAT-like domain of OGA from the simplest eukaryote *Trichoplax adhaerens*. This protein has 30% sequence identity to the human HAT-like domain and can serve as a model for studying its function.

### (57) Compositional change of N-glycan profile in response to chronic exposure to low dose Ionizing radiation in Medaka

Yeni N. Perez-Gelvez[1], Simone Krurz[1], Michael Tiemeyer[1], Olin E. Rhodes[2], Carl Bergmann[1] and Gerardo Gutierrez-Sanchez[1]

*[1]Complex Carbohydrate Research Center, University of Georgia; [2]Savannah River Ecology Laboratory, University of Georgia*

Over the last 70 years, the presence of Ionizing radiation (IR) in our daily lives has increased due to its use in medicine, agriculture, nuclear power, and the development and testing of weapons. As a consequence, the presence of background radiation (radiation that we receive from our environment) has increased as well. On average, we receive 2.3 mGy/year from background radiation. Gamma radiation, a form of IR, has enough energy to remove an electron from its nucleus, affecting at different levels the biological matter. IR can cause breaks in DNA by direct action as well as protein oxidation, lipid peroxidation, inactivation of enzymes etc. due to the presence of reactive oxygen species. The consequences will depend mainly on the dose and type of exposure. At high doses (> 1 Gy) the consequences have been the focus of many studies. However, at low levels of IR, the effects are not well understood. The overall aim of the project is to better understand how an organism responds to chronic exposure (low doses over a prolonged time) of IR. We have detected compositional changes in the N-glycan profiles of Medaka fish carcasses and organs in response to different low dose levels of IR. Increased expression of multiantenary structures and increased glycan fucosylation were detectable, in agreement with previous studies that analyzed high doses of radiation in radiotherapy patients. The modification in the expression of N-glycans as result of the different levels of low dose of IR suggests a role for glycosylation in response to irradiation at low levels and indicates that N-glycans may serve as biomarkers for low dose IR.

### (58) Metabolic pathway analysis that combines glyco-gene transcript analysis with glycan structural data derived from differentiated human stem cell lineages

Alison V. Nairn[1], Harrison Grace[1], Katelyn Rosenbalm[1], Melina Galizzi[1], Mitche dela Rosa[1], Mindy Porterfield[1], Michael Kulik[2,3], J. Michael Pierce[1,2], Stephen Dalton[2,3], Michael Tiemeyer[1,2] and Kelley W. Moremen[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*

We are studying human embryonic stem cell (hESC) development as a model for changes in glycan-related gene transcripts and glycan structural changes that occur during human stem cell differentiation. Transcript abundance of ~900 glycan-related genes was profiled by a combination of high-throughput qRT-PCR and RNA-Seq to identify changes that occur during stem cell development, including those that may interact with or regulate glycan-related gene expression. The long-term goals are to identify changes in transcript abundance for glycan biosynthetic and catabolic genes, assign these transcript changes to metabolic pathway steps, and correlate the data with parallel analysis of glycan structures derived from mass spectrometry analysis. The ability to analyze and visualize changes within and between two disparate analytical data types is challenging because the forms of the resulting data and the scaling of the data are quite different. In addition, changes in key regulatory enzyme abundance may lead to changes in glycan flux at pathway endpoints that are remote from the respective regulated pathway step. We are developing a pathway visualization tool to address this issue through the use of the Metscape plug-in associated with the open source software Cytoscape. This visualization framework for pathway analysis allows the creation of intuitive biosynthetic pathways where changes in both glycan structures derived from MS analysis and changes in transcript abundances determined by RNA-Seq and qRT-PCR can be highlighted. The use of a unified platform for the display of both data types allows the identification of correlative changes that occur during cellular differentiation. The pathway visualization framework is being applied to the analysis of glycan structural data and transcript abundance for undifferentiated H9 human ES cells as well as H9 cells differentiated into cardiac progenitors (WT-1), smooth muscle (SM), a hepatic lineage (Liver) and neural crest (NC). This set of differentiated cell lineages encompasses all three germ layers; endoderm (Liver), mesoderm (WT-1 and SM) and ecotoderm (NC) and will highlight germ layer-specific changes in glycan-related gene expression and glycan structural information. (supported by NIH grant P41GM103490 to JMP)

### (59) The zebrafish galectin DrGRIFIN displays specificity for blood group B oligosaccharides and participates in early development of the eye lens

Chiguang Feng[1], Jinyu Wang[1], Núria Gonzalez-Montalbán[1], Prasun Guha[1], Hafiz Ahmed[2] and Gerardo R. Vasta[1]

[1]*Department of Microbiology and Immunology;* [2]*Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, UMB, Institute of Marine and Environmental Technology, Baltimore, Maryland, USA*

Galectins, a family of β-galactoside-binding lectins, have key roles in early development and tissue regeneration. Our prior studies have shown that they are involved in retinal regeneration using the zebrafish as a model system, and ongoing studies in our laboratory are aimed at elucidating their functions in eye lens development. Through *in silico* analysis we identified in zebrafish a proto type galectin sequence (which we designated DrGRIFIN) that in BLAST analysis produced the highest match with the mammalian GRIFIN (Galectin related inter fiber protein). Like the mammalian equivalent, DrGRIFIN is expressed in the lens fiber cells, as revealed by whole mount *in situ* hybridization and immunostaining of 2 dpf (days post fertilization) embryos. Unlike the mammalian homologue, DrGRIFIN contains all amino acids critical for binding to carbohydrate ligands and its activity was confirmed as the recombinant DrGRIFIN could be purified to homogeneity by affinity chromatography on lactosyl-Sepharose, and showed strong binding to glycoproteins such as asialofetuin and porcine stomach mucin (PSM) that was effectively inhibited by lactose. Glycan array analyses (CFG at NCFG; and ABH at Core H, CFG) revealed that DrGRIFIN has a striking specificity for blood group B oligosaccharides (types 1, 2 and 6), a property shared with other galectins that display a short loop between the β strands S4 and S5. ELISA and SPR measurement confirmed that DrGRIFIN binds to asialofetuin and PSM, as well as neoglycoproteins displaying blood group B oligosaccharides, but not to those with blood group A oligosaccharides. In preliminary studies we investigated the effect of knocking down the expression of either DrGRIFIN or DrGal1-L2, using morpholino (MO)-derived antisense oligonuclotides to block the protein translation. The GRIFIN-specific MO suppressed the protein expression up to 90% at 24 hpf in protein extractions. We observed a phenotypical change consisting of a diffuse smaller lens in up to 65% of the animals in MO group, as compared to that in the mismatch control group. Ongoing studies in our laboratory are aimed at using CRISPR/CAS9 to knockout GRIFIN to enable further analysis of the resulting phenotype. [Supported by grant R01GM070589 from the National Institutes of Health to GRV. We are grateful to Dr. Richard D. Cummings, Dr. David Smith and Dr. Jamie Heimburg-Molinaro, Core H-CFG and NCFG, for glycan array analysis].

### (60) Does metabolic state regulate Notch trafficking by modulating its O-glycan modifications?

Kenjiroo Matsumoto and Robert S. Haltiwanger

*Complex Carbohydrate Research Center, The University of Georgia*

Notch is a transmembrane receptor that mediates cell-cell interactions and controls various cell-fate specifications in metazoans. Notch is modified by *O*-glycans in the ER and Golgi, and transported to the plasma membrane where the Notch extracellular domain (NECD) binds to its ligands (Delta-like-ligand1, 4/Jagged1,2) resulting in Notch activation. The NECD contains 36 epidermal growth factor-like (EGF) repeats. Serine/Threonine residues within the $C^1$-X-S-X-(P/A)-$C^2$ and $C^2$-X-X-X-X-(S/T)-$C^3$ sequences in EGF repeats are modified with *O*-glucose and *O*-fucose glycans, respectively. *O*-Glucose is added by Protein *O*-glucosyltransferase 1 (POGLUT1), and *O*-fucose by Protein *O*-fucosyltransferase 1 (POFUT1). Although Notch function is seriously compromised in single *Drosophila POFUT1 (Ofut1)* or *POGLUT1 (rumi)* mutants, Notch trafficking is mostly normal. However, in double mutants of *Drosophila POFUT1* and *POGLUT1*, Notch accumulates inside of the cells (Ishio et al., 2015, JBC, 2;290(1):505-19) and Notch is not presented on the cell surface (Matsumoto et al., 2016, JBC, M115.710483.), further reducing Notch activity. Similar results were observed in mammalian *POGLUT1* and *POFUT1* double mutant cells (Takeuchi et al., 2017, JBC, M117.800102.). These results suggest that *O*-fucose and *O*-glucose modifications of Notch work together in Notch trafficking from ER to cell membrane. Since nutrient levels affect donor nucleotide sugar levels in cells, we wanted to know whether varying glucose levels in the extracellular environment would alter Notch glycosylation and trafficking. Using HEK293T cells, we found that Notch is not efficiently presented on the cell membrane and accumulates inside of the cells when the extracellular glucose level is low. We then analyzed the glycosylation status of the Notch protein isolated from the medium or accumulated inside of the cells by mass spectrometry. We found that *O*-fucose and *O*-glucose sites are modified at high stoichiometry on Notch isolated from medium and produced under normal conditions., In contrast, *O*-glycan stoichiometry on EGF repeats is only partial on Notch protein from cell lysates, and the stoichiometry is reduced when cells are grown in low glucose conditions. These result suggest that Notch glycan modifications are diminished in low glucose conditions, resulting in Notch trafficking defects in human cells *in vitro*. We are checking whether this Notch trafficking phenotype under low glucose conditions also occurs in *Drosophila in vivo*.

### (61) Mapping the Glycoproteome with Activated Ion Electron Transfer Dissociation

Nicholas M. Riley[1,2], Alexander S. Hebert[1], Michael S. Westphall[1] and Joshua J. Coon[1,2,4]

[1]*Genome Center of Wisconsin, University of Wisconsin–Madison, Madison, WI, 53706, USA;* [2]*Department of Chemistry, University of Wisconsin–Madison, Madison, WI, 53706, USA;* [3]*Department of Biomolecular Chemistry, University of Wisconsin–Madison, Madison, WI, 53706, USA;* [4]*Morgridge Institute for Research, Madison, WI, 53706, USA*

Protein glycosylation is a prevalent, chemically complex, and biologically diverse post-translational modification (PTM) involved in a wide array of intra- and inter-cellular functions. Approximately half of all expressed proteins undergo glycosylation, and this heterogeneous modification accounts for the greatest proteome diversity over any other PTM. Changes in protein glycosylation are associated with cellular proliferation, inter-cellular communication, and metabolic processes, making the characterization of the cellular landscape of protein glycosylation integral to advancing our understanding of cell biology. Glycan microheterogeneity, i.e., different glycans modifying the same glycosite, makes glycan identity at a given site crucial to the biological context of the modification. This unique feature of glycosylation makes analysis of intact glycopeptides imperative for glycoproteome characterization, but current analytical tools are ill-suited for this task.

Tandem mass spectrometry (MS) is an ideal platform to advance glycoproteomic technology, but current dissociation methods are often suitable only for characterization of either peptide or glycan moieties. This mandates multiple analyses of the same precursor ions that limit throughput and challenge data interpretation. We have developed a tandem MS dissociation method called activated ion-electron transfer dissociation (AI-ETD) that addresses several of challenges of intact glycopeptide analysis. Through the use of concurrent ion-ion reactions and infrared photo-activation, AI-ETD can access glycan and peptide information from intact glycopeptides in a single MS/MS scan, and with this AI-ETD provides (1) improved product ion generation for peptide backbone sequencing, (2) higher MS/MS success rates to sequence more glycopeptides per experiment, and (3) valuable fragmentation for glycan composition determination. Here we showcase AI-ETD for large-scale intact glycopeptide characterization on the newest generation of Orbitrap instruments. We show that >2000 localized N-glycosites can be confidently identified from approximately 30,000 localized N-linked glycopeptide spectral matches (>7,500 unique) in mouse brain tissue. This represents a more than 3-fold increase over recent studies in the number of glycopeptides and glycosites that can be identified via intact glycopeptide analyses.

### (62) Identification of Two Novel Protein O-glucosyltransferases that Modify Notch EGF Repeats

Daniel Williamson[1], Hideyuki Takeuchi[1], Michael Schneider[2] and Robert Haltiwanger[1,2]

[1]*Complex Carbohydrate Research Center, University of Georgia;* [2]*Department of Biochemistry and Cell Biology, Stony Brook University*

Notch signaling is essential for proper development of multicellular organisms. In humans, dysregulation of Notch signaling has been implicated in several cancers and other diseases such as CADASIL (Cerebral Autosomal-Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy) and Alagille syndrome. Glycosylation of the Notch receptor is integral for the regulation of Notch signaling and activation. So far, three major forms of *O*-glycosylation are known to occur at predicted consensus sequences within the epidermal growth factor-like (EGF) repeats of the extracellular domain of Notch: *O*-glucosylation, *O*-fucosylation, and *O*-GlcNAcylation. Glycosyltransferases Protein O-glucosyltransferase 1 (POGLUT1), POFUT1, and EOGT are responsible for these modifications, respectively. A recent co-crystal between a portion of Notch1 and Delta-like ligand 4 (DLL4) revealed an additional hexose modification on EGF11 at a novel site that interacts with DLL4. EGF11 has previously been implicated in the binding interface of Notch and its ligands, potentially linking this novel modification to the regulation of Notch signaling. We have identified two homologs of POGLUT1, POGLUT1 and POGLUT2, and through radioactive enzyme activity assays have shown that these enzymes add glucose to this novel site on EGF11. To further confirm these two enzymes are responsible for this modification, single and double knockout HEK293 cells were generated using CRISPR/Cas9 gene editing. Mass spectral analysis of mouse Notch1 EGF extracellular domain expressed in these cells confirmed loss of the glucose modification on EGF11 when both POGLUT2 and POGLUT3 were knocked out. We performed rescue experiments with either POGLUT2 or POGLUT3, and confirmed the restoration of the glucose modification on EGF11 of mouse Notch1. The site of modification appears to be conserved on Notch3 and Notch4, but not Notch2. Elimination of the novel *O*-glucose site on EGF11 by mutagenesis reduces Notch1 activity, suggesting that POGLUT2 and POGLUT3 are novel modulators of Notch activity. These results lay the groundwork for future studies on these two enzymes in order to enhance our understanding of Notch *O*-glycosylation and how it regulates Notch signaling, as well as identifying additional proteins these novel enzymes may be modifying. This work was supported by NIH grant GM061126.

### (63) Production of double mutants that lack paralogue enzyme genes for mucin-type glycan biosynthesis

Naosuke Nakamura[1], Kasumi Tsukada[1], Yuki Tsujimoto[1], Yoshiaki Nakayama[2], Morichika Konishi[2] and Akira Kurosaka[1]

[1]*Department of Molecular Biosciences, Kyoto Sangyo University;* [2]*Microbial Chemistry lab., Kobe Pharmaceutical University*

Mucin-type O-glycosylation is one of the major post-translational modifications of membrane and secreted proteins. Among glycosyltransferases involved in mucin-type glycan biosynthesis, polypeptide alpha-N-acetylgalactosaminyltransferases (GalNAc-Ts) are particularly important because they catalyze

the initial step of O-glycosylation and determine the number and positions of glycans in proteins. The *GALNT* genes that code for GalNAc-T consist of a large gene family with 20 isozymes in humans, and their biological functions largely remain unclear. We have been analyzing their functions with focus on a *GALNT* subfamily, so-called Y-subfamily, that occurs specifically in vertebrates.

We previously isolated all zebrafish *galnt* genes including Y-subfamily genes, *galnt8, galnt9, galnt17, galnt18a* and *galnt18b* and demonstrated their expression patterns in the embryos (1). We found that isozymes with high expression in mammalian brains were also highly expressed in the zebrafish brain. Recently, we established mutant zebrafish lines of Y-subfamily, *galnt9* and *galnt18a*, with TALEN and found that they did not show significant phenotypic alterations during the early embryonic development. As to enzymes with a large gene family, apparently normal phenotypes of the mutants may be ascribed to the concurrent expressions of other isozymes in the same tissue, and multiple knockout/knockdown of isozyme genes may be required to investigate their functions.

Zebrafish has 17 *galnt* genes, and some of them have overlapping expression patterns in a tissue. In addition, zebrafish has two paralogue *galnt18* genes, *galnt18a* and *galnt18b*. *In situ* hybridization analysis of the paralogues indicated partially overlapping expression patterns, suggesting their functional redundancy. To produce double mutants that lack both *galnt18a* and *galnt18b*, we designed single guide RNA (sgRNA) targeting to a highly homologous region between *both* genes. By microinjecting a Cas9 protein and the sgRNA into embryos, we successfully generated double hetero mutants for each paralogue gene at F1 generation. We then mated the F1 double-hetero mutants, and obtained F2 embryos with genotypes of single (either *galnt18a* or *galnt18b*), and double (*galnt18a/18b*) knockout mutants. Although all genotypes were obtained at theoretical Mendelian ratios in the embryonic stage, *galnt18a/18b* double mutant failed to grow into adult fish. This indicated that coordinate expressions of *galnt18a* and *galnt18b* may be required for normal growth to adults in zebrafish. We are investigating molecular mechanism that causes early death of the mutants and also analyzing endogenous substrates of *galnt18a/18b*.

**Reference**

1. Gene Expression Patterns, Y. Nakayama *et al.* 2014. **16**(1):1–7.

### (64) Neural activity regulates neural-specific glycosylation

Sarah Baas Robinson, Nickita Mehta, Katherine H. Tiemeyer, Debora Witkowski, Peng Zhao, Lance R. Wells and Michael Tiemeyer
*CCRC, University of Georgia*

Neural-specific protein glycosylation is an essential characteristic of neuronal development and neuronal plasticity. However, the mechanisms that regulate neural-specific glycosylation remain largely unknown. We have taken advantage of readily available genetic and biochemical tools to identify pathways responsible for regulating the neural-specific expression of a family of core difucosylated N-linked glycans known as HRP-epitopes in the *Drosophila* embryo. In a forward genetic screen we recovered a mutation (designated ms16) in an ubiquitin ligase (*roc2*) that results in decreased HRP-epitope expression but increased abundance of a subset of complex N-glycans. These differential glycomic alterations in *roc2*$^{ms16}$ embryos imply that the mutation impacts the organization and fidelity of the secretory pathway as opposed to simply attenuating core fucosylation. Characterization of early and late Golgi markers revealed increased overlap in ms16mutants, consistent with the hypothesis that altered Golgi compartmentation broadly impacts the access of glycan processing enzymes to glycoprotein substrates. Since the Roc2 ubiquitin ligase is expected to alter the abundance of multiple proteins, we performed LC-MS/MS based proteomic analysis to identify normally ubiquitinated proteins whose abundance is increased in *roc2*$^{ms16}$ embryos. Among the candidate substrate proteins for Roc2 ubiquitin ligase, we detected a significant increase in the expression of the Na + /K + -ATPase alpha subunit (ATPalpha). To assess whether increased abundance of the ATPalpha protein directly impacts protein glycosylation, we employed genetic and pharmacologic approaches to reduce ATPalpha function. We compared expression of HRP-epitopes in wild type adults, *roc2*$^{ms16}$ homozygous adults, and a balanced deletion line heterozygous for complete loss of ATPalpha by ELISA. While *roc2*$^{ms16}$ adults were greatly reduced in HRP-epitope expression compared to wild type, adults from the deletion line exhibited enhanced total HRP-epitope expression. To directly address whether the ATPase function of the ATPalpha protein was essential for regulating neural-specific glycosylation, we optimized methods for treating embryos with a well-characterized inhibitor of ATPalpha. Treatment of embryos with ouabain significantly increased HRP-epitope expression in both wild type and mutants, demonstrating that increased ATPalpha activity drives the *roc2*$^{ms16}$glycosylation phenotype. Therefore, we hypothesize that neural activity, which is absolutely dependent on the activity of the Na + /K + -ATPase, influences the functional architecture of the Golgi apparatus and, as a consequence, the appearance of neural-specific glycans at the neuronal cell surface. Furthermore, our optimized approach to pharmacologic treatment of living, intact *Drosophila* embryos offers opportunities to screen other small molecules and other genotypes toward the goal of understanding regulated glycoprotein glycosylation.

### (65) Regulation of Mixed Lineage Leukemia 1 (MLL1) by O-GlcNAc Modification

Ilhan Akan and John A. Hanover
*LCMB, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health*

The Mixed Lineage Leukemia 1 (MLL1) protein is an important epigenetic factor in leukemia. Mutations in the MLL1 gene are not only associated with nearly 10% of Acute

Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL), but also common among solid tumors. The most common MLL1 mutations are chromosomal translocations that fuse the part of the MLL1 gene in frame with over 60 other genes, creating novel fusion proteins. Leukemias that have the MLL1 translocations are characterized by poor prognosis. MLL1 protein is a histone methyltransferase acting on histone 3, lysine 4. The MLL1 protein is cleaved to form an N-terminal and C-terminal peptides that bind to each other to form the functional protein. MLL1 maintains cell type specific gene expression and cellular memory. We seek to understand the role of nutrient-derived intracellular signaling molecule UDP-GlcNAc on epigenetic machinery. Since tumor cells are in constant need for nutrients, understating the role that the nutritional signaling play in cells will help us understand and treat cancer. Two cellular enzymes are in charge of connecting nutritional input to cytoplasmic and nuclear proteins. O-GlcNAc Transferase (OGT) uses UDP-GlcNAc and transfer GlcNAc onto the hydroxyl groups of serine or threonine residues (termed O-GlcNAc), while O-GlcNAcase (OGA) removes GlcNAc from the same residues. Many of the sites are also phosphorylated or very close to phosphorylation sites. In many cases O-GlcNAcylation and phosphorylation plays opposite roles in altering the activity of their targets. We are therefore interested in how nutrient-derived O-GlcNAcylation of MLL1 effects it's function that could contribute to tumorigenesis. We previously showed that the MLL1 ortholog in *Drosophila*, Trithorax (TRX) is regulated by *O*-GlcNAc. In this study, we discovered that MLL1 is modified by nutrient-sensor *O*-GlcNAc in human cancer cell lines. Furthermore, OGT interacts with MLL1. Host Cell Factor-1 (HCF1) protein is essential for the interaction between OGT and MLL1. Furthermore, our results suggest that MLL1 regulates OGT expression. Moreover, our results suggest that increasing cellular O-GlcNAcylation levels increased levels of cyclin A and cyclin E during the G1/S phase of cell cycle progression. Based on our results, we hypothesize that the MLL1 protein may auto-regulate it's activity by controlling the levels of OGT that adds the nutrient-sensor O-GlcNAc modification on MLL1. Increased MLL1 levels that is a signature of MLL translocation could therefore increase O-GlcNAc levels in tumors carrying MLL1 translocations. Increased O-GlcNAc could then significantly contribute to tumorigenesis. Targeting this relationship could provide a novel potential therapeutic for tumors with MLL1 translocations.

### (66) Glycoproteoform Network Analysis (GNA) On Top-Down MS (TDMS) Datasets

Steven M. Patrie[1,2,3]
[1]*Northwestern University;* [2]*Department of Chemistry;* [3]*Proteomics Center of Excellence*

Glycans exist in sequential biosynthetic reaction networks driven by diverse glycosyltransferases in the secretory pathway. For TDMS utilizing a high-resolving power MS1 event intact proteins are often observed with more than one N-glycan. The mass differences between adjacent spectral proteoforms don't relate to sequential processing predicted by reaction networks, precluding use of glycoproteoforms (gp) in clinical proteomics investigations for biomarker discovery. To address this, GNA assigns spectral products to plausible gp reaction paths generated from permutations of 100's of glycans. Our model enables: 1) MS1 spectra annotation when gp are subjected to multidimensional separations (p*I*, mass, and hydrophobicity), 2) quantitative assessment of distinct glycosyltransferase activities from MS1 data, and 3) probability-scoring in intact gp informatics.

GNA considers N-glycans at different positions as a generic glycan and tracks the number of fucose(F), hexose(H), GlcNAc (GN), and sialic acid(SA) residues present. Permutations of $g_p$ for mono- to tetra- glycoproteins were used to generate network diagrams that support qualitative/quantitative evaluation of MS1 data. 25–100 µg of protein (standards, cerebrospinal fluid (CSF), and cell-lysates) was analyzed by offgel isoelectric focusing (IEF) and nano-RPLC-FTMS on a LTQ-Orbitrap-XL/ETD. IEF fractions were collected from 0.1–0.4 p*I*intervals. Modeling and data processing were automated in MATLAB (Mathworks). Data were converted to proteoform (p) vectors (mass($p_i$), pI($p_i$), RT($p_i$), int($p_i$)) with pre- and post-deisotoping S/N, fitting, and multidimensional binning parameters examined to reduce noise.

We previously discovered >200 intact L-PGDS proteoforms in CSF (pmic.201300368). Using available software, 100's of man-hours were required to link related proteoforms in LCMS data for a single proteome (24 IEF fractions). Implementation of GNA has reduced multidimensional data processing to < 1 hr. For a glycoprotein harboring two N-glycan sites (e.g., L-PGDS), permutations of the 417 common N-glycan structures predicted from sequential biosynthetic reaction networks in the secretory pathway (hyrbrid and complex up to tetra-antennary) yields 11237 unique proteoforms of which 1284 have distinct generic glycan composition ($g_p$). Each $g_p$ has unique mass with 97.5% resolvable at 40 ppm mass accuracy (~1 Da at 22,500 Da). For tri- and tetra-glycosylated proteins increased Fuc/SA overlap was mitigated by inclusion of observed p*I* into matching metrics. We next developed network plots to examine glycoproteofom hierarchy and perform differential expression analysis across IEF-SPLC-FTMS datasets and between disease cohorts. Filtering of 1284 $g_p$ products by the number of GlcNAcs provided 9 networks that each contain $SA_n$ and $Fuc_n$ branches with branch length associated $Hex_n$. For TDMS studies, accurate mass assignment was used to link observed proteoforms to network maps andto link $g_p$ paths to MS1 spectra.

### (67) Fucosylated chondroitin sulfate oligosaccharides exert anticoagulant activity by targeting at intrinsic tenase complex with low FXII activation: Importance of sulfation pattern and molecular size

Junhui Li[1], Shan Li[1], Shiguo Chen[1], Xingqian Ye[1], Donghong Liu[1], Robert J. Linhardt[2] and Tiani Ding[1]
[1]*Zhejiang University;* [2] *Rensselaer Polytechnic Institute*

***Background***: Two fucosylated chondroitin sulfates(fCSs) were isolated from sea cucumber, *Isostichopus badionotus* and *Pearsonothuria graeffei*. Fragmatation of these fCSs afforded oligosaccharides that were purified and their mechanism of anticoagulation was studied.

***Methods***: Fenton reaction followed gel filtration chromatography afforded fCS oligosaccharides that were identified by mass and NMR spectroscopy. *In vitro* anticoagulant activities were measured by activated partial thromboplastin time (APTT) and thrombin time (TT), thrombin and factor Xa inhibition, and activation of factor XII.

***Results***: Oligosaccharides from trisaccharide to nonasaccharide and pentadecasaccharide were prepared and purified from both fCSs. Free radicals preferentially acted on GlcA residues without affecting other groups. The inhibition of thrombin and factor X activity of the fCSs oligosaccharides by antithrombin III (AT III) and heparin cofactor II (HCII) were affected by their molecular weight and fucose branches. Oligosaccharides with different sulfation pattern of the fucose branch had the similar effect on inhibiting the FXa by the intrinsic factor Xase (factor IXa-VIIIa complex). Oligosaccharides with 2,4-O-sulfo fucose branches from fCS-*Ib* showed higher activity than ones with 3,4-O-disulfo branches obtained from fCS-*Pg*. A heptasaccharide was identified as the minimum size required for anticoagulation. Moreover, factor XII activation was inhibited after the molecular size decreased to a nonasaccharide.

***Conclusion***: The Fenton reaction selectively degrades fCSs and generates a series of well-defined oligosaccharides. The resulting fCSs oligosaccharides exert anticoagulant activity only in the intrinsic coagulation pathway. Molecular size and fucose branch sulfation are important for anticoagulant activity and reduction of size can reverse the activation of FXII caused by native fCSs.

***General importance***: We clarify the anticoagulant mechanism of fCSs oligosaccharides, helping in the development of fCS-derived anticoagulant drugs and their quality control.

### (68) Extraction of Novel RG-I enriched pectin from mandarin citrus peel

Hua Zhang, Jian le Chen, Jun hui Li, Xing qian Ye and Shi guo Chen
*Zhejiang University*

Pectin is extensively used as thickener and gelling agent in the food industry. It contains multiple "building blocks" mainly including linear-form homogalacturonans (HG), highly branched-form rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). However, the commercial pectin is mainly extracted from citrus peel under high temperature and strong acid, which results in debranching of RG-I and RG-II region, and thus the final highly esterified pectin is rich in HG domain and performs well in gel formation. The obtained pectin can be subsequently treated with base to get lowly esterified pectin also rich in HG domain.

However, recent studies have widely confirmed the importance of the RG-I in pectin with higher biological activities. Thus, the present study developed novel extraction methods for extraction of pectin by sequence treatments of mild acid extraction followed by alkaline treatment. The reaction temperature was controlled at 28 °C to avoid debranching of the pectin, and lowly esterified pectic polysaccharides rich in RG-I domain can simultaneously be obtained. The yields of polysaccharides extracted by acid(PA) and by base(PB) were 4.22 wt% and 18.93 wt%, respectively. And monosaccharides assay indicated PA contained 57.43% galacturonic acid while PB contains only 23.37%. Rha/GalA indicated PA and PB consisted mainly of RG-I structure as the result values were 0.10 and 0.49, respectively. The GPC-MALLS results indicated that both were spherical molecules with a molecular weight of 281.7 kDa and 743.2 kDa, respectively. Further NMR and FT-IR analysis indicated that they were typical pectic polysaccharides, and the esterification degree was 55.66% for PA while the PB was non-esterified. The AFM images directly displayed their branched-chain morphology, which were consistent with the results by monosaccharides determination. Rheological and gel analysis showed that the two polysaccharides had good thickening and gelling properties and could be potentially used in food.

### (69) Suppressive effects of bisecting GlcNAc on terminal modifications of N-glycans

Yasuhiko Kizuka[1], Miyako Nakano[2] and Naoyuki Taniguchi[1]
[1]*Disease Glycomics Team, RIKEN;* [2]*Graduate School of Advanced Sciences of Matter, Hiroshima University*

Bisecting GlcNAc, a branched structure of N-glycan highly expressed in brain, is biosynthesized by glycosyltransferase GnT-III. By analyzing GnT-III (Mgat3) knockout mice, we previously showed that bisecting GlcNAc promotes Alzheimer's disease pathology (Kizuka et al., EMBO Mol. Med., 2015). However, the physical functions of bisecting GlcNAc are still unclear.

Previous biochemical studies revealed that bisected N-glycans do not serve as substrates for other branching enzymes, such as GnT-IV and GnT-V. Furthermore, recent structural and simulation studies suggest that the presence of bisecting GlcNAc impacts on global conformation of N-glycans. These findings suggest that bisecting GlcNAc can globally modulate N-glycan structures, but thishypothesis has not been tested in vivo yet.

In this study, we first analyzed N-glycan structures in GnT-III knockout brain by LC-MS, and found complete loss of bisected structure. Surprisingly, we discovered that the terminal modifications with fucose and sialic acid are greatly upregulated in knockout brain. Moreover, the level of a

major terminal epitope of N-glycan, HNK-1, also increased. These results indicate that various terminal modifications of N-glycan are upregulated by deleting bisecting GlcNAc. As an underlying mechanism, we revealed that almost all the biosynthetic enzymes for terminal modifications, including fucosyltransferases, sialyltransferases and HNK-1-related enzymes, commonly have poor activity toward N-glycan substrateswith bisecting GlcNAc. These findings indicate that bisecting GlcNAc suppresses various terminal modifications of N-glycans through enzymatic properties.

### (70) Time-resolved N-glycan processing allows a functional resolution of the Golgi in CHO cells

Ilaria Affolter[1], Chia-Wei Lin[1], Ernesto Scibona[1], David Brühlmann[2], Jonathan Souquet[2], Hervé Broly[2] and Markus Aebi[1]

*[1]ETH Zürich, Switzerland; [2]Merck, Switzerland*

In the synthesis of glycoproteins, the glycan portion is not template-driven as for the protein backbone, and it depends on a multitude of factors. In the endoplasmic reticulum (ER), a defined glycan structure (G3M9N2) is transferred onto selected asparagine residues. The processing of glycans by different enzymes, in the ER and subsequently in the Golgi, yields a site-specific, heterogeneous glycan on the secreted product. The reasons that lead to such microheterogeneity are still poorly understood. In fact, our current knowledge on N-glycosylation are limited to the spatial localization of the enzymes *in vivo* and the reactions carried out by them *in vitro*. However, the understanding of the chronology of *in vivo* N-glycosylation is missing.

The goal of this project is to monitor the N-glycosylation process *in vivo* in a time-resolved manner. The use of newly developed, mass spectrometry based methods in glycoproteomics enables on one side to gain site-specific glycan structure information of the protein of interest. On the other side, by applying a chase regime using stable isotope labelling of amino acids in cell culture (SILAC) time-resolved information on the N-glycosylation are obtained.

Data showing the N-glycan distribution of an IgG (model protein) and the time-resolved N-glycan processing (from ER, through the Golgi, to secretion) will be presented. With the help of a mathematical model, a precise delineation of the chronology of IgG N-glycosylation is gained. This enables a functional resolution of the ER and Golgi that goes beyond the spatial localization of the enzymes.

A quantitative description of the intracellular N-glycosylation network will be the basis for the design of productions conditions that result in a defined N-glycoprotein product, such as biosimilar.

### (71) Bio-orthogonal fluorescent tags for carbohydrate analysis and neoglycolipids-based functional assays development

Katarzyna Brzezicka, Matthew Allen and Sarah Allman

*Chemical Glycobiology Laboratory, School of Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK*

Heterogeneous populations of structurally complex carbohydrates mediate fundamental biological processes, such as fertilization, signal transduction and pathogen recognition events. However, even though the importance of glycosylation has been known for decades, due to the non-template driven biosynthesis of sugars and their challenging chemical synthesis, the accessibility of these information rich structures is limited.[1]

In this regard, we believe, that isolation of glycans from readily available biological material and their use in development of functional assays can be crucial to decipher structure-function relationship of different glycoconjugate molecules. Here, we will present our recent efforts to develop novel fluorescent tags bearing bio-orthogonal modifications enabling chromatographic separation of glycans obtained from natural sources and their downstream uses in functional assays, including glycan microarrays. Our labels will be based upon commercial tags including procainamide, 2-anthranilic acid (2-AA) and2-aminobenzamide (2-AB),[2] to ensure compatibility with accepted analysis workflows. These tags will be further diversified with additional modifications, such as alkyne or norbornene moieties. These biorthogonal linkers will further enable conjugation of labelled glycans with appropriately functionalised ligation partners *via* copper catalysed alkyne-azide cycloaddition or *via* catalyst-free inverse electron demand Diels-Alder reactions.[3]

On the example of simple carbohydrates, we will show labeling performance of novel labels compared to commercial tags and their further conjugation to lipids resulting in neoglycolipids for microarray or liposomes preparation. Subsequently, selected tags will be used to label heterogeneous mixtures of complex *N*-glycans released enzymatically from model glycoproteins and cultured cells and used to study carbohydrate-protein interactions.

We believe that developed fluorescent bio-orthogonal tags will provide exciting methodology for utilisation of natural glycan resources, generation of complex neoglycoconjugates and functional assays, making carbohydrates more accessible to the wider scientific community and facilitating study of biological functions of glycans.

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### (72) Early steps in the initiation of clustered O-glycosylation impact final glycan heterogeneity: Implications for autoantigen formation in a chronic kidney disease

Tyler J. Stewart[1,2], Kazuo Takahashi[1,2,3], Milan Raska[1,4], Robert H. Whitaker[2], William J. Placzek[2], Matthew B. Renfrow[2] and Jan Novak[1]

[1]*Department of Microbiology, University of Alabama at Birmingham;* [2]*Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham;* [3]*School of Medicine, Fujita Health Univsersity, Toyoake, Japan;* [4]*Department of Immunolgy, Palacky University Olomouc, Czech Republic*

Mucin-type *O*-glycosylation is a common post-translational modification that occurs in the Golgi complex. These *O*-glycans are synthesized in a stepwise non-template driven process, starting with the addition of *N*-acetylgalactosamine (GalNAc) to Ser or Thr residues by a family of twenty GalNAc-transferases (GalNAc-Ts) in humans. The glycans are often added in a highly clustered manner to Ser-, Thr-, and Pro-containing repeat regions of secreted or membrane proteins. Despite the potential complexity of glycosylation products derived from the stepwise non-template driven process, *O*-glycans of a given protein usually exhibit a restricted heterogeneity as it concerns the sites of glycosylation and glycan heterogeneity at a given site. In the autoimmune disease IgA nephropathy (IgAN), the restricted heterogeneity of *O*-glycans on IgA1 hinge region (HR) is altered in a manner that produces a glycan autoantigen(s) recognized by anti-glycan autoantibodies. Analysis of these glycans in IgA1 from IgAN patients revealed increased site-specific galactose deficiency (Gd) as well as altered density of HR *O*-glycans. We hypothesized that changes in glycosylation initiation by GalNAc-Ts could contribute to the changes of IgA1 HR *O*-glycans consistent with autoantigen formation. GalNAc-T2 has been proposed to be the predominant GalNAc-T responsible for IgA1 HR glycosylation. We investigated the process of GalNAc-T2 glycosylation of IgA1 HR peptides using high-resolution LC-MS with ETD to determine mechanisms by which GalNAc-Ts can alter HR glycans in a manner consistent with the IgAN glycophenotype. Our results showed that GalNAc-T2 can start at four of the nine potential sites of glycosylation within IgA1 HR, two of which are often sites of Gd in IgAN. IgA1 HR contains two partially overlapping ten amino-acid repeats; two dominant first glycosylation sites occurred at identical Thr residues within these repeats. The amino acids surrounding these Thr residues closely resemble the glycosylation motif identified for the catalytic domain of GalNAc-T2. We found that the lectin domain of GalNAc-T2 played a key role in increasing the total density of glycans added to IgA1 HR, but it did not alter first site selection. The second site of glycosylation was impacted by the first site of glycosylation, and for each initial site of glycosylation multiple second sites were observed. Analysis of the kinetics of glycosylation associated with each of the four initial sites of glycosylation showed that the initial site of glycosylation affected the final glycan density of IgA1 HR. Together, our results demonstrate that GalNAc-T2 follows a semi-ordered pathway of glycosylation. The lectin domain of GalNAc-T2 plays a critical role in site selection and density of IgA1 HR *O*-glycans. Initial glycosylation events by GalNAc-Ts as early as first glycan addition can alter the hinge region *O*-glycans in a manner consistent with IgAN through mechanisms of site selection and glycan density.

## (73) Polysaccharide similarities: extractable glycan oligomers and glycosylated protein cores of glycogen, starch and cellulose

Allen K. Murray[1,2]

[1]*HIBM Research Group, Inc.;* [2]*Glycan Technologies, Inc.*

Common characteristics of the glucan polymers glycogen, starch and cellulose include glycan oligomers extractable by mild acid and glycosylated protein cores. Initially glycan oligomers were extracted from developing cotton fibers. Their abundance correlates with the onset of cellulose synthesis in the secondary wall of cotton fibers. In developing cotton fibers which produce the glycan oligomers *in vitro* assays have shown oligomer production to be inhibited by tunicamycin which also suggests a biosynthetic role. These oligomers are ß-glucans with the inclusion of inositols, iditol, sorbitol, mannose and galactose which are present at one end of the glucan. The oligomers can not be completely degraded by cellulases. Acid hydrolysis of the isolated cellulose fraction of mature cotton fibers releases several oligosaccharides which contain the same carbohydrates as well as amino acids. Subsequently, analogous glycan oligomers have been extracted from glycogen and starch by the same method. In the case of starch gel following their release a glycosylated protein residue remains which contains inositols, iditol, mannose, galactose and glucose. The glycan oligomers released from starch are a series of maltooligosaccharides and have been reported to be possible biosynthetic intermediates in starch synthesis. The oligomers are also released on cooking of starch excised from nixtamalized corn. They are believed to play a role in the binding of masa after cooking. A glycosylated protein residue has been found on degradation of starch with amyloglucosidase. A similar glycosylated protein residue is also obtained on degradation of glycogen with rhGAA (lysosomal α-glucosidase) which also contains inositols, iditol, sorbitol, mannose, glucose and galactose. The comparative biochemistry of the analogous glycosylated protein cores of starch, glycogen and cellulose is of interest with respect to the biosynthesis of these polysaccharides and their enzymatic degradation. In the case of glycogen, the inability of rhGAA to completely degrade glycogen raises questions related to enzyme replacement therapy in Pompe disease which is caused by a deficiency of GAA. It was previously shown that isolated rat liver lysosomal α-glucosidase followed by phosphorylase and debrancher can not completely degrade glycogen either. In cotton the glycosylated protein core of cellulose may play a role in the resistance to biodegradation. (Supported by Genzyme GZ-2017-11679, 2015-11412,and Cotton Incorporated 98-638, 12-231)

### (75) Exploring the specificity of chemical tools for O-GlcNAc labeling

Michelle R. Bond, Pamela D. Cook, Carolyn C. Woodroofe, Rolf E. Swenson and John A. Hanover
*National Institutes of Health*

The dynamic posttranslational modification of serine/threonine residues of cytoplasmic and nuclear proteins with O-linked N-acetylglucosamine (O-GlcNAc) has been shown to be a critical factor for cellular processes including signal transduction, epigenetic regulation, stress response, protein degradation, and apoptosis. Manipulation of the proteins that are involved with the addition (O-GlcNAc transferase, OGT) and removal (O-GlcNAcase, OGA) of the modification by inhibitors and genetic means has become routine for understanding the implications for changes in global O-GlcNAcylation on these cellular processes. Moreover, the use of unnatural, bio-orthogonal sugars known to label O-GlcNAc have become a popular way to modify and monitor proteins of interest.

We were interested to define the incorporation of both azide and alkyne functionalized bio-orthogonal sugars taking advantage of metabolic oligosaccharide engineering. Herein, we focus on the use of acetylated GlcNAc and GalNAc analogs, $Ac_4$-GlcNyne and $Ac_4$-GalNyne. The unnatural sugars were metabolically incorporated via the GlcNAc biosynthetic pathway and we used Cu(I)-catalyzed [3 + 2] azide–alkyne cycloaddition (CuAAC) reaction to monitor the sugars' incorporation.

We took a multi-pronged approach to discern the cellular molecules labeled by the bio-orthogonal sugars including genetic and chemical tools. (1) GlcNAc hydrolases were mutated to compensate for the additional N-acetyl group length and we monitored cleavage of fluorescent alkynyl sugar derivatives *in vitro*. Cleave of the unnatural sugars were also monitored in cellular lysates using multiple glycan-specific enzymes. (2) Chemical tools such as OGT and OGA inhibitors as well as beta-elimination were used to indicate whether sugars were O-linked. (3) Genetic tools including RNAi and genetic ablation were used in concert with other methods to support our findings.

In this way, we have established a strategy to define the specificity of incorporation into the various glycan classes. Our studies reveal that a substantial fraction of the CuAAc signal from GlcNyne- and GalNyne-treated cells are resistant to beta-elimination and hexosaminidase treatment. We suggest that the fraction observed to be incorporated into O-GlcNAc is dependent upon at least the length of metabolic incorporation. The findings suggest that multiple parameters should be considered when interpreting the results of metabolically incorporated unnatural sugars as surrogates for the intracellular O-GlcNAc modification.

### (76) Glycoproteomics for high-throughput characterization of mammalian proteoglycans

Alejandro Gomez Toledo[1], Waqas Nasir[2], Jonas Nilsson[2], Fredrik Noborn[2], Jeffrey D. Esko[1] and Goran Larson[2]
[1]*Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA, USA;* [2]*Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden*

Proteoglycans are acidic and heavily modified glycoproteins that are essential for development, tissue organization and cell hemostasis. They are linked to pathogenesis by modulating microbial infection, cancer behavior and cardiovascular dysfunction. To assess their impact on human health and disease, a deep understanding of their structure and function is required. Unfortunately, progress has been severely hampered by analytical difficulties related to their glycosaminoglycan (GAG) chains. Here, we developed glycoproteomics workflows for the structural characterization of mammalian chondroitin sulfate and heparan sulfate proteoglycans from body fluids and cells. We combined enzymatic degradation steps, chromatographic separation and high-resolution mass spectrometry. Additionally, we developed SweetNET, a bioinformatics platform to cope with the large amounts of data generated from these high-throughput experiments. In addition to the structural characterization of known human proteoglycans, we conducted detailed site-specific analysis of 21 novel core proteins carrying GAG modifications. They included several human pro-hormones, defining them as a novel class of proteoglycans. We also identified unique GAG modifications and developed protocols for the analysis of hybrid proteoglycans. We are currently refining these promising approaches to address the role of heparan sulfate proteoglycans in infectious diseases, and more specifically in the context of sepsis.

### (77) Generation of a complex-type multi-antennary N-glycan microarray to define recognition patterns of N-glycan binding partners

Chao Gao[1], Lauren A. Byrd-Leotis[1,2], Melinda S. Hanes[1], Richard H. Banes[1], Tanya McKitrick[1], Nan Jia[1], David A. Steinhauer[2] and Richard D. Cummings[1]
[1]*Department of Surgery, Beth Israel Deaconess Medical Centre, Harvard Medical School;* [2]*Department of Microbiology, Emory University School of Medicine*

*N*-glycosylation is a type of complex and yet exquisitely controlled post-translational modification of glycoproteins. *N*-glycans can modulate properties of glycoproteins such as conformation, solubility, stability, turnover and antigenicity. More importantly, through recognition by their glycan-binding partners, *N*-glycans are implicated in many biological and pathological events. In spite of their functional significance, it has been difficult to define the molecular mechanisms by which these *N*-glycans exert their functions, partially due to a lack of a library of well-characterized standards and a high-throughput platform. Here we used an integrated chemoenzymatic approach to efficiently generate a library of

complex-type multiantennary *N*-glycans. A biantennary sialylated glycopeptide (SGP), purified from egg yolk powder, was converted by a series of chemoenzymatic reactions to an Fmoc-labelled *N*-glycosylasparagine core structure with GlcNAc-Asn residues at the non-reducing terminal. A set of human recombinant glycosyltransferases was then used to elongate the core to yield a library of 32 multi-antennary *N*-glycoasparagine isomers in sub-milligram quantities with different numbers and linkages of sialic acid (Neu5Ac). We also demonstrated that these compounds after de-Fmoc protection can generate free reducing-glycans by oxidative release and be printed on NHS surfaces to fabricate glycan microarrays. Using the prepared glycan microarrays, we observed interesting and characteristic branch binding preferences of many plant lectins such as ConA, L-PHA, DSL, LCA, and others. We also observed differential binding patterns among various strains of influenza viruses and between Siglecs 1 and 9. In conclusion, our strategy provides a convenient approach for production of highly complex *N*-glycan isomers and is an excellent platform to investigate the recognition of *N*-glycan binding partners. [This work was supported by CEIRS grant contract HHSN272201400 to DS and RDC and NIH Grant P41GM103694 to RDC. We thank Dr. Kelley Moremen (glycoenzymes.ccrc.uga.edu) and the Repository for providing the glycosyltransferases and the NIH grants P41GM103390 and P01GM107012].

### (78) The expanding glycouniverse: diverse glycan modifications in lower eukaryotes

Iain B. Wilson, Katharina Paschinger, Alba Hykollari, Jorick Vanbeselaere, Shi Yan and Barbara Eckmair
*Universität für Bodenkultur*

The classical view has been that N-linked oligosaccharides in 'lower' eukaryotes (protists, fungi, plants and invertebrates) are simple. However, recent data shows that, other than plants, the diversity of N-glycan modifications in non-vertebrate species is extremely high. From our own studies on *Dictyostelium*, *Trichomonas*, *Penicillium, Acanthamoeba*, mosquitoes, moths and other insects, *Caenorhabditis* and other nematodes, molluscs and planaria, a wide range of new glycan modifications have been demonstrated using an off-line HPLC/MALDI-TOF-MS workflow. Particularly underrepresented in older studies are the 'charged' modifications of N-glycans from lower eukaryotes, such as glucuronic acid, sulphate, phosphoethanolamine, aminoethylphosphonate and phosphorylcholine. In addition, unusual galactosylation, fucosylation and mannosylation events (on the core and bisecting positions) increase the range of possibilities. Thus the concept of 'simple' organisms possessing only 'simple' glycans is no longer a true reflection of the actual glycosylation capacity of invertebrates. The coming years are certain to result in further glycomic surprises, certainly reflecting the presence of an unexplored treasure chest of Golgi enzymes in the analysed species, but also with repercussions for the use of invertebrate cell lines as expression hosts.

### (79) A novel fluorescent bifunctional linker for glycan derivatization

Mohui Wei, Tanya McKitrick, Robert Kardish, Jamie Heimburg-Molinaro, Lijun Sun and Richard D. Cummings
*Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA 02215*

Glycan microarrays are one of the key technologies for functional glycomics studies. Shotgun glycomics is a strategy for functional glycomics study of structurally undefined natural glycans, which involves releasing, derivatization, purification and printing of natural glycans from biological sources to generate shotgun glycan microarrays, followed by interrogation of the glycan microarrays with glycan binding proteins and characterizing functionally important glycans. A key step in the Shotgun glycomics process is the derivatization of glycans, which enables separation and quantification of diverse natural glycans from biological sources, as well as printing the glycans on a microarray surface. We designed and synthesized a novel cleavable fluorescent bifunctional linker, which can efficiently derivatize free reducing glycans and form intact close-ring reducing end, which is especially essential for some glycans to retain epitope integrity. This linker can also be facilely cleaved to regenerate free glycans for structural analysis by Mass spectrometry. A library of structural defined glycans were derivatized by both the novel linker and AEAB, and printed to generate a close-ring reducing end glycan microarray (the linker tagged) and an open-ring reducing end glycan microarray (AEAB tagged). The generated glycan microarrays were interrogated by various Glycan-Binding Proteins (GBPs) to show the comparable binding between AEAB-tagged and our novel linker-conjugated glycans. The opportunity to have closed-ring glycans printed on arrays is a robust addition to our glycan array toolbox, especially for smaller sized glycans.

### (80) Recognition of glycosaminoglycans by human galectin-3: Mechanism of binding and possible functional complexities due to dual specificities

Tarun Dam, Christina Welch, Melanie Talaga, Ni Fan and Purnima Bandyopadhyay
*Mechanistic Glycobiology, Department of Chemistry, Michigan Technological University*

We have recently shown that human lectin galectin-3 (Gal-3) binds to sulfated glycosaminoglycans or GAGs (heparin and chondroitin sulfates) and chondroitin sulfate proteoglycans (CSPGs). Heparin was found to be a monovalent ligand of Gal-3, while chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) and CSPGs were able to cross-link Gal-3 through multivalent interactions. The present study reports (i) the molecular basis of GAG recognition by Gal-3 and (ii) intra-GAG and GAG-glycoprotein competition for Gal-3. (i) Binding studies and data analysis suggest that Gal-3 interacts with sulfated GAGs (CSA and CSC) and CSPGs through a mechanism called "bind and jump". In this mechanism, Gal-

3 first binds to the linear structures of CSA and CSC, dissociates (jumps) temporarily and then rebinds. This process is repeated several times and then Gal-3 completely dissociates from GAGs after several rebinding. The number of rebinding and hence the residence time of Gal-3 on GAGs increases with the length of the GAGs. As residence time is proportional to affinity, longer GAGs have higher affinity for Gal-3 than their shorter counterparts. Cross-linking ability of GAGs is also proportional to their length. (ii) Our data show that GAGs and proteoglycans compete among themselves as well as with glycoproteins for Gal-3. These data suggest that known glycoprotein-Gal-3 and GAG-GAGBP (GAG-binding proteins, such as growth factors) interactions in biological systems may not be as bipartite as they seem. In the close proximity of a biological system where all entities (Gal-3, Gal-3 specific glycoproteins, GAGs, proteoglycans and GAGBPs) are present, glycoprotein-Gal-3 interactions will be influenced by competitive GAGs and proteoglycans, GAG/proteoglycan-GAGBP interactions will be interrupted by competitive Gal-3, and GAG/proteoglycan-Gal3 interactions will challenged by GAGBPs and Gal-3-speci?c glycoproteins. Therefore, the known glycoprotein-Gal-3 and GAG/proteoglycan-GAGBP interactions might be far more complicated than they currently appear.

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### (81) Glycosyltransferases that assemble the repeating unit of the intestinal pathogen Escherichia coli O104:H4

Inka Brockhausen[1], Diana Czuchry[1] and Walter A. Szarek[2]
[1]*Department of Biomedical and Molecular Sciences and* [2]*Department of Chemistry, Queen's University, Kingston ON, Canada*

*Escherichia coli* serotype O104:H4 (ECO104) is a potent intestinal pathogen that causes bloody diarrhea and hemolytic-uremic syndrome. An outbreak in Germany with more than 3000 people infected demonstrated the need to understand how these bacteria synthesize their virulence factors. The outer O-antigenic polysaccharides linked to lipopolysaccharides (LPS) interact with the environment and are important virulence factors. The O antigen of ECO104 consists of repeating units with the structure:

[4Galα1-4Neu5,7,9Ac3 α2-3Galβ1-3GalNAcβ1-]. This oligosaccharide contains an unusual linkage of Galα1-4 to Neu5Ac not found in human glycoconjugates, but also the structural mimics of the human T and sialyl-T-antigen. The O antigen gene cluster of ECO104 includes genes encoding enzymes that synthesize Neu5Ac, flippase Wzx, and polymerase Wzy, indicating that the O antigen is assembled by the polymerase pathway. In addition, three glycosyltransferase (GT) genes, *wbwA, wbwB* and wbwC are present. We have characterized the three glycosyltransferases, β1,3-Gal-transferase WbwC (GT2 family), α2,3-sialyltransferase WbwA (GT100) and α1,4-Gal-transferase WbwB (GT4), and used them to synthesize the O104 repeating unit oligosaccharide. As expected, WbwABC are specific for their donor substrates and the oligosaccharide structures of their acceptor substrates and sequentially assemble the O104 antigen repeating unit. However, it is surprising that all of these GTs require the diphosphate group of their acceptors which are based on chemically synthesized GalNAcα-diphosphate-phenylundecyl. These enzymes thus have unique specificities. Mutagenesis revealed amino acids in WbwC, WbwA and WbwB that are essential for activity. To inhibit these GTs we synthesized series of compounds based on structural variations of hydrophobic imidazolium salts. This work identifies potential antibacterial targets and a strategy for vaccine synthesis.

### (82) A new UDP-hexose/UDP-HexNAc 4-epimerase from the archaeon *Methanococcus maripaludis*

Sulav Sharma, Yan Ding, Ken Jarrell, Inka Brockhausen
*Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, Canada*

*Methanococcus maripaludis* are archaea that are motile through their archaellum. This complex motility structure is composed of N-glycosylated glycoproteins (archaellins). The N-glycans are complex tetrasaccharides linked to Asn residues of archaellins with the structure: 4-deoxy-5-O-Me-ManNAcβ1-4ManNAc3NAmA[Thr-6]β1-4GlcNAc3NAcAβ1-3GalNAcβ-Asn. Most of the enzymes that assemble this N-glycan or synthesize the nucleotide donor substrates have not yet been studied. Mutations of the *mmp1090* gene showed that it plays an essential role in the biosynthesis of the fourth sugar residue at the non-reducing end. The Thr residue attached to the third sugar of these N-linked tetrasaccharides is also dependent on *mmp1090*. However, *mmp1090* gene deletion did not affect the presence of GalNAc, the first sugar attached to Asn. Sequence analysis indicate that *mmp1090* encodes a UDP-Glc 4-epimerase. However, the 4-epimerase activity and enzymatic properties of MMP1090 have not been studied. We have developed novel coupled and sensitive assays to determine the production of UDP-sugars by purified MMP1090. For each of the nucleotide sugar substrates UDP-Glc, UDP-Gal, UDP-GalNAc and UDP-GlcNAc, we used a coupled glycosyltransferase reaction that would irreversibly convert the 4-epimerase product to a glycosyltransferase product. MMP1090 4-epimerase activity showed specificity towards UDP-Glc, UDP-Gal, UDP-GlcNAc and UDP-GalNAc without the addition of metal ion or $NAD^+$ cofactors. However, MMP1090 was unable to use synthetic GalNAcα-diphenyl-undecyl or GlcNAcα-diphenyl-undecyl as a substrate. Mutations of the Tyr and Lys residues of the conserved YxxxK motif showed that these residues are essential for activity. The results are of significance to understand the synthesis of complex N-glycans and the factors controlling motility of *M. maripaludis*.

### (83) New software for glycan array for data processing, storage and presentation

Yukie Akune[1], Sena Arpinar[2], Mark Stoll[1], Lisete M. Silva[1], Angelina S. Palma[3], Yan Liu[1], René Ranzinger[2] and Ten Feizi[1]

[1]*Glycosciences Laboratory, Department of Medicine, Imperial College, London, UK;* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA;* [3]*UCIBIO-Faculty of Science and Technology, NOVA University of Lisbon, Portugal*

The field of glycan arrays has been evolving since 2002. This technology is powerful for elucidating carbohydrate-protein interactions and assigning the ligands among the increasing numbers of glycans arrayed.

Our laboratory has developed a glycan array data processing software suite for carbohydrate microarray data management, to include imaging, storage, processing and presentation [1]. To date, two databases and six data management software tools have been developed in phases using Microsoft Office and Visual Studio. More than 160 experimental results are available published using this software [2].

Here we will be presenting work now underway to develop a professional software suite, based on Java and Eclipse, to replace the existing in-house Microsoft Office-based microarray software [1], and to make it a freely available cross-platform standard tool for the scientific community. This is based on the modular GRITS Toolbox [3], a software system originally developed for processing, interpretation and archiving of glycomic mass spectrometry data. The GRITS Toolbox incorporates important additional features for storing glycan structures and other metadata such as project information, sample description and details of the experiments performed. By implementing the glycan array software as a module (plugin) of the toolbox, we are capitalizing on these pre-existing functions. Users will be able to store the data with filtering, sorting, tabulation and chart generation functions including textual and symbolic presentations of glycans for data presentation, archiving and data sharing. We will also implement a function to generate reports conforming to MIRAGE guidelines for glycan microarray-based data [4].

This project is supported by The Wellcome Trust.

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### (84) Fine-tuning limited proteolysis – A novel role for regulated site-specific O-glycosylation in ß1-Adrenergic Receptor cleavage and function

Christoffer K. Goth[1], Hanna E. Tuhkanen[2], Hamayun Khan[2], Shengjun Wang[1], Yoshiki Narimatsu[1], Lasse H. Hansen[3], Christopher Overall[4], Henrik Clausen[1], Katrine T. Schjoldager[1] and Ulla Petäjä-Repo[2]

[1]*Copenhagen Center for Glycomics, Department of Cellular and Molecular Medicine, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.;* [2]*The Medical Research Center Oulu, Research Unit of Biomedicine, University of Oulu, P. O. Box 5000, FI-90014 Oulu, Finland.;* [3] *Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital, DK-2100 Copenhagen Ø, Denmark;* [4]*Centre for Blood Research, Department of Oral Biological and Medical Sciences, and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada*

Increasing evidence positions site-specific GalNAc-type O-glycosylation (hereafter O-glycosylation) catalysed by the large family of UDP-GalNAc polypeptide GalNAc-transferases (GalNAc-Ts) as a general co-regulator of limited proteolysis. We and others have previously demonstrated a general role for site-specific O-glycosylation in co-regulating proprotein processing. Subsequently, we have systematically investigated the role of site-specific O-glycosylation in ADAM mediated ectodomain shedding and found compelling evidence for a general co-regulatory role for site-specific O-glycosylation. Wefurther validated our findings in a cell line model system and a knockout mouse using TNF-a as an example. Very recently, we identified the first example of a G-Protein Coupled Receptor (GPCR) to be co-regulated by site-specific *O*-glycosylation mediated by a unique GalNAc-T isoform. Using glycoengineered cell lines and a GalNAc-T2 knock-out rat model we show that GalNAc-T2 specifically glycosylates the human $\beta_1$-adrenergic receptor $\beta_1$AR N-terminus, including the Ser-49 residue at the location of the common S49G single-nucleotide polymorphism. We demonstrate that impairedO-glycosylation and enhanced proteolysis lead to attenuated receptor signaling, because the maximal response elicited by the βAR agonist isoproterenol and its potency in a cAMP accumulation assay were decreased in HEK293 cells lacking GalNAc-T2. The results provide a new level of $\beta_1$AR regulation that may pave the way for new therapeutic strategies for cardiovascular diseases. Furthermore, we have now identified more than 60 GPCRs carrying N-terminal O-glycosylation which in several cases overlap with identified cleavage sites. This opens up the exciting possibility that other GPCRs are co-regulated in a similar manner and suggest a broader interplay between GPCR N-terminal O-glycosylation, cleavage and function.

### (85) Onco-Golgi: the role for Golgi disorganization in MGAT5-mediated progression of prostate cancer

Armen Petrosyan[1,3,4] and Chad A. LaGrange[2]

[1]*Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center;* [2]*Division of Urologic Surgery, Department of Surgery, University of Nebraska Medical Center;* [3]*The Nebraska Center for*

*Integrated Biomolecular Communication; [4]The Fred and Pamela Buffett Cancer Center*

The Golgi apparatus is undergoing significant remodeling in cancer cells, and it is linked to both aggressiveness and survival of tumor. It is known that in N-glycans the typical bi-antennary N-linked chain can be transformed into a β1,6-branched structure by the action of N-acetylglucosaminyltransferase-V (MGAT5). Overexpression of MGAT5 was detected in different types of tumor and suggested as a prognostic marker in prostate cancer (PCa), however, the mechanism is unknown. MGAT5 is antagonized by N-acetylglucosaminyltransferase-III (MGAT3), the enzyme that modifies glycans by the addition of a bisecting GlcNAc and whose enhanced expression has been found to suppress metastasis. Intriguingly, Matriptase (Matr), one of the key plasma membrane-bound and metastasis-specific proteases, is a client protein for MGAT5, and MGAT5-glycosylation induces Matr binding to Galectin-3 (Gal-3). In a normal prostate and in low passage LNCaP (c-24) cells, which represents androgen responsiveness, the Golgi appears as a compact structure localized in the juxtanuclear space, and both MGAT5 and MGAT3 is detected in the Golgi. Predictably, adenocarcinoma cells from Gleason grade 2–4 prostate tumors exhibited fragmented Golgi, and the distribution of these enzymes was asymmetric, with MGAT5 still predominantly in the Golgi, but MGAT3 in the cytoplasm. We revealed that mislocalization of MGAT3 in aggressive PCa cells is determined by impaired dimerization of its Golgi docking partner, giantin. In the meantime, MGAT5 is still able to reach the Golgi, because it employs the alternative Golgi targeting mechanism, the complex GRASP65-GM130. Importantly, in LNCaP (c-24) cells, Matr bears glycans modified by MGAT3, but not MGAT5, in spite of their both intra-Golgi localization. Further analysis by 3D Structured Illumination Microscopy revealed that MGAT3 is distributed mainly in the proximal Golgi, while MGAT5 was detected in the distal Golgi compartments. Conversely, in androgen-restrictive high passage LNCaP (c-85) and PC-3 cells, N-glycans of Matr was modified by MGAT5. In normal prostate tissue and LNCaP (c-24) cells, Matr and Gal-3 were detected in both the perinuclear area and cytoplasm, while in tissue from PCa patients and LNCaP (c-85) and PC-3 cells, both proteins were found predominantly at the cell periphery. This suggests that Golgi disorganization and MGAT5-catalyzed N-glycans are the prerequisites for the efficient link of Matr to Gal-3 and subsequent retention of Matr at the cell surface. Finally, we found that 72 h exposure of LNCaP (c-24) cells to physiological levels of ethanol seems to mimic the phenotype of highly aggressive PCa cells. In sum, our data explore the mechanistic understanding of the role of the Golgi complex in the development of PCa, as well as establish a link between alcohol consumption and progression of PCa. (The work is supported by the Nebraska Center for Integrated Biomolecular Communication Systems Biology Core NIGMS P20-GM113126).

## (86) Analysis of the interaction between GBPs and glycans using the MCAW web tool

Masae Hosoda, Yushi Takahashi and Kiyoko F. Aoki-Kinoshita
*Department of Bioinformatics, Graduate School of Engineering, SOKA University*

Many experimental data have been made available in several databases such as KEGG GLYCAN [1], the Consortium for Functional Glycomics (CFG) [2], Glycosciences.de [3] and JCGGDB [4]. Bioinformatics techniques to analyze these experimental data are needed. In the CFG and Lectin frontier Database (LfDB) [5], data of interaction results between glycan binding proteins (GBP) and glycans are accessible to the public on the web. LfDB stores quantitative interaction data of binding constants of lectin-pyridylaminated glycans by automated FAC-FD experiments.

For elucidation of glycan binding-recognition patterns by GBPs, we have developed the MCAW (Multiple Carbohydrate Alignment with Weights) [6] tool which can represent the common substructures among a set of glycan structures. The MCAW algorithm is based on the KCaM algorithm [7], which aligns pairs of carbohydrate structures, and ClustalW [8], which is a popular multiple amino acid sequence alignment algorithm. The MCAW tool has now been implemented on the web as a part of RINGS [9] to output a multiple glycan alignment of a set of glycan structures.We prepared and analyzed datasets of glycan structures that exhibit affinity for GBPs from CFG and LfDB.

As a result of these analyses, we found that glycans bound by PL (Potato lectin)/ STL (*Solanum tuberosum* lectin), which is a chitin-binding lectin, contain GlcNAc as a common component and is highly aligned, reflecting the known fact that it interacts with GlcNAcβ1-4 structures. WGA (Wheat Germ Agglutinin) is known to bind to GlcNAc residues, and the WGA glycan dataset resulted in a GlcNAc disaccharide aligned 100%. In mouse galectin-4, Galβ1-4, Fucα1-2, and Galα1-3 are highly aligned, which determine blood type B. These results correlate with the suggested binding recognition pattern of mouse galectin-4.

We show that our tool can be used to analyze results from two different types of experiments. Although these experiments have results of different affinity values and use different approaches for interaction analysis, the Glycan-GBP interaction affinity from the CFG data and LfDB data could be aligned and representative recognition patterns could be obtained. Therefore we have also developed a database to summarize all our analysis of the binding data in CFG and LfDB using our MCAW tool.

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### (87) Analysis of Highly Sialylated and Low-Input Glycoprotein Samples on the GlycanAssure™ System

Wenjun Zhou, Shaheer Khan, Raymond Lee, Natalee Gautam, Jenkuei Liu, Bharti Kunnummal, Peter Bell and Kyle R. Gee
*ThermoFisher Scientific*

Glycosylation plays many important roles in biological processes involving function, pharmacokinetics, stability, and immunogenicity, which is why it is important to monitor recombinant protein heterogeneity to ensure safety, effectiveness, as well as consistency in manufacturing for biopharmaceuticals. The most widely used method in laboratories today for glycan analysis has the disadvantage in that sample prep is time consuming with overnight N-Glycan release steps, additional purification steps to remove excess salts, excess labeling reagents, large protein input, and difficulty producing data on highly sialylated glycans.

Because sialylated glycans elute at the back end of the LC profile, analysis and accurate quantitation of highly sialylated glycans consistently is a challenge in LC methods. CE offers an advantage in analysis as sialylated glycans migrate at the front end of the electropherogram, making it an ideal platform to analyze and accurately quantitate highly sialylated glycans. CE also offers an opportunity to analyze samples with low glycoprotein input as sample requirement is very low, in comparison to typical LC methods.

In this study, we evaluated APTS-labeling conditions on highly sialylated glycans, specifically Fetuin. Furthermore, we also tested 3500xl CE detection capability for analysis of glycans at low sample input and labeling with APTS dye. Results reinforce the robustness of the GlycanAssure workflow, even with shorter labeling time and low protein input. The 3500xl CE platform achieves quantitative, high resolution glycan analysis, with high-sensitivity and high-reproducibility.

### (88) Integration of Glycoscience Data in GlyCosmos Using Semantic Web Technologies

Issaku Yamada[1] and Kiyoko F. Aoki-Kinoshita[2]
[1]*The Noguchi Institute;* [2]*Soka University*

Collaborative research by researchers in various fields is indispensable in life science research, and in order to elucidate glycan function, cooperation of researchers with a variety of expertise is also necessary. As a foundation for integrating glycoscience research, we have started developing the Glycoscience Portal called GlyCosmos. This portal uses the glycan structure notation WURCS [1] and the GlycoRDF ontology [2] as the standard formats for representation of glycan information. In addition to the already developed International Glycan Structure Repository GlyTouCan [3], plans to develop a new Glycoconjugate Structure Repository GlyComb and a curated GlyCosmos Database are in place. The GlyCosmos Database will store curated glycan-related information such as molecular structures, glyco-genes, pathways, etc.

In this presentation, we introduce the outline of the GlyCosmos Portal and introduce our attempt to connect GlyCosmos Database with GlycoNAVI [4] to integrate small molecules and glycans, mainly in collaboration with Protein Data Bank Japan (PDBj) [5] and PubChem [6,7]. In addition, we will present work on the visualization and analysis of glycan structures in PDB data.

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### (89) LLO Hydrolysis Is Selectively Catalyzed By the Stt3B-OST Complex

Hua Lu[1], Charles S. Fermaintt[2], Nan Yan[2] and Mark A. Lehrman[1]
[1]*Department of Pharmacology;* [2]*Departments of Immunology and Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA*

The oligosaccharyltransferase (OST) complex mediates protein *N*-glycosylation in the ER lumen. Mammalian cells express two OST complex isoforms, with the STT3A or STT3B catalytic subunits. Substantial amounts of free oligosaccharides (fOS) are released by OST due to hydrolysis of lipid-linked oligosaccharides (LLO). This process is generally considered stochastic, with no known purpose. Whether one or both mammalian OST isoforms catalyze LLO hydrolysis is unknown.

We used well-characterized HEK293-derived CRISPR-Cas9 knockouts lacking either STT3A or STT3B to evaluate the basis of LLO hydrolysis by fluorophore-assisted carbohydrate electrophoresis. OST function was assayed in cells specifically permeabilized at the plasma membrane by the bacterial

pore-forming toxin anthrolysin O, to preserve fragile ER protein complexes and membrane organization. Both knockouts grew similarly to HEK controls, with no obvious changes in global N-glycan profiles, sugar mono/bis-phosphates, nucleotide-sugars, or steady state LLO compositions.

Both OST isoforms demonstrated classical oligosaccharyltransferase activity toward an acceptor tripeptide, as anticipated. HEK control and STT3A (-/-) cells (both containing STT3B) also had robust LLO hydrolysis activities that rivaled their transferase activities. Unexpectedly, essentially no hydrolysis activity was detected in STT3B (-/-) cells expressing only the STT3A isoform. Some fOS were increased in STT3A (-/-) cells compared to HEK controls. They were cytosolic, and experiments with OST inhibitor NGI-1, LLO synthesis inhibitor tunicamycin, and glycosidase inhibitors kifunensine and castanospermine suggested the fOS originated from LLO by OST action, not by ER-associated degradation. The reason for their accumulation in STT3A (-/-) cells is unclear.

Previously, we reported that mutation of ER-associated TREX1 (DNase III; a suppressor of autoimmunity) stimulates OST-dependent LLO hydrolysis. We took advantage of fortuitously low expression of TREX1 in HEK293 cells to restore TREX1 by transfection into HEK293-derived cells. Reintroduction of TREX1 significantly decreased fOS levels in HEK control and STT3A (-/-) cells, but not in STT3B (-/-). These results implicate the STT3B-OST complex as a functional target of TREX1 for control of LLO hydrolysis.

In summary, the STT3B-OST complex catalyzes LLO hydrolysis, and this activity is suppressed by TREX1. STT3A-OST has no detectable LLO hydrolysis activity, but its absence indirectly increases cytosolic fOS resulting from LLO hydrolysis by STT3B-OST. Greater understanding of the interplay of STT3A, STT3B, and TREX1 may help explain the etiologies of STT3A-CDG, STT3B-CDG, and TREX1-deficiency.

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### (90) How Prior Glycosylation Modulates the Specificity of the ppGalNAc-Transferases: Mechanisms Underlying Remote and Neighboring Glycosylation

Earnest James Paul Daniel[1], Matilde de las Rivas[2], Erandi Lira-Navarrete[2,3], Ramon Hurtado-Guerrero[2] and Thomas A. Gerken[1]

*[1]Depts of Biochemistry and Pediatrics Case Wstern Reserve University, Cleveland OH; [2]BIFI, University of Zaragoza, Zaragosa, Spain; [3]Presently: Copenhagen Center for Glycomics, Univ. Copenhagen, Denmark*

Polypeptide GalNAc-transferases (ppGalNAc-Ts) initiate mucin-type O-glycosylation by transferring N-acetylgalactosamine (GalNAc) onto the hydroxyl groups of Ser and Thr residues of target substrates. There are ~20 ppGalNAc-T isoforms in mammals with most isoforms showing differential spatial/temporal expression patterns. Aberrant expression of individual isoforms has been associated with several diseases including cancers. The very large number of isoforms suggests that substrate selection and Thr/Ser site preferences are likely to differ among isoforms, thereby orchestrating mucin type O-glycosylation.

Almost all GalNAc-Ts contain an N-terminal catalytic domain adopting a GT-A fold and a C-terminal β-trefoil fold lectin domain connected by a flexible linker. Previous studies from our lab using randomized glycopeptide substrates have shown the presence of long range/remote glycopeptide activity in these transferases mediated by the lectin domain, i.e. a prior GalNAc-O-Ser/Thr is recognized by the lectin domain that subsequently directs remote (>6 residues) glycosylation in either an N- or C- direction. For example, ppGalNAc-T4 recognizes N-terminally placed remote GalNAc while ppGalNAc-T2 recognizes C-terminal placed remote GalNAc. How these isoforms prefer a particular N- or C- prior glycosylation is not well understood. In addition, several of these enzymes present a short range/neighboring glycopeptide activity (<5 residues), which is dependent on the catalytic domain and differs among isoforms depending on the N- or C-terminal placement of the prior GalNAc. For example ppGalNAc-T2 lacks this activity while ppGalNAc-T4 recognizes a GalNAc-O-Ser/Thr directly N-terminal of the acceptor site.

Here we report, kinetic and structural studies on linker domain chimeras of ppGalNAc-T2 and ppGalNAc-T4. Kinetic studies of these enzymes against a series of N- or C-terminal glycopeptide substrates reveal that the flexible linker dictates the transferase's long range glycopeptide preferences. This is consistent with our structural studies of wild type ppGalNAc-T2 and wild type T4 bound to glycopeptide substrates which show the lectin domain in different positions relative to the catalytic domain.

Finally, we present crystal structures of GalNAc-T4 with bound glycopeptides that provide the molecular basis of the glycosylation in a lectin and catalytic dependent manner, and reveal residues likely important for GalNAc recognition in both binding sites. Kinetic studies on such catalytic and lectin domain mutants, against an array of glycopeptide substrates, confirm the residues involved in GalNAc binding. Overall this work for the first time fully reveals the molecular basis of both the long and short range glycosylation preferences of the GalNAc-Ts.

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### (91) Analytical Services and Trainings at the Complex Carbohydrate Research Center

Roberto N. Sonon, Asif Shajahan, Ian Black, Justyna Dobruchowska, Stephanie Archer-Hartmann, Bernhard Jaehrig, Artur Muszynski, Radnaa Naran, Sara Porfirio,

Nitin Supekar, Zhirui Wang, Dandan Zhou, Christian Heiss and Parastoo Azadi
*CCRC-University of Georgia*

The **CCRC Analytical Services Laboratory** at The University of Georgia offers services for structural characterization of glycoconjugates derived from animal, plant, and microorganisms. Our scientists have many years of experience on designing/conducting a wide array of experiments in the area of Glycobiology to cater the objectives of researchers. The service laboratory is equipped with state-of-the-art instruments such as Thermo Orbitrap-Fusion MS, Velos Orbitrap-Elite MS, LTQ-Orbitrap MS, AB SCIEX TOF/TOF 5800 MALDI-MS, Bruker MALDI-TOF MS, CE-MS, NMR, HPLC, HPAEC, and GC-MS. We present examples of glycoconjugate analyses that utilize one or a combination of techniques. Briefly described below are some analyses for each class of glycoconjugate.

Glycoproteins: glycopeptides and peptides analysis, release of N-and O-linked glycans from purified, gel-prepared, cell or crude tissue glycoproteins; mapping N-and O-linked glycosylation sites, identification of type of N-linked glycans, i.e. biantennary, triantennary, tetraantennary, high mannose, hybrid or complex; determination of residue and linkage composition and their sequence in oligosaccharides, ring size, anomeric configuration, and determination of points of attachment, and linkage of non-carbohydrate constituents such as phosphate and sulfate.

Glycosaminoglycans (GAGs): isolation of GAGs from cells and tissues, depolymerization, analysis and quantification of disaccharide building blocks (quantification of HS/CS/HA, CTA-SAX, molecular weight determination, degree of sulfation, isolation and sequencing of individual oligosaccharides by MS and NMR).

Polysaccharides: determination of glycosyl composition and linkage, ring size, and anomeric configuration; and purification to homogeneity by SEC and/or ion exchange chromatography.

Lipopolysaccharides (LPS): isolation of LPS directly from bacterial cells, release of Lipid A from O-antigen, and characterization of Lipid A by MS and O-antigen by techniques used for polysaccharides.

Peptidoglycan: isolation and purification of murein/peptidoglycan from Gram-positive and Gram-negative bacteria, determination of amino acid and glycosyl composition, analysis of muropeptides by LC-MS, structural characterization by high resolution mass spectrometry (MALDI-TOF-MS, NSI-MS).

Lipids and glycolipids: determination of fatty acid composition of ceramide, diacylglycerol or triacylglycerol, and phospholipids.

We also offer annual hands-on training courses on **techniques for structural characterization of glycoproteins, polysaccharides, and GAGs**. On these trainings, participants perform experiments and analyze end products by mass spectrometry, GC/MS, HPLC-UV and fluorescent detection, HPAEC-PAD, lectin blotting, and thin layer chromatography. The hands-on experience is further enhanced by lectures from experts/scientists on various fields of glycobiology. In addition to the above-mentioned trainings, we offer a separate mass spectrometry course for glycoproteins.

## (92) Mechanism of Neuropilin-2 polysialylation: Does the autopolysialylation have a cameo?

Gaurang P. Bhide, Ninoshka R.J. Fernandes, Joseph L. Zapater and Karen J. Colley
*Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60607*

Polysialic acid (polySia) is a unique, oncodevelopmental glycopolymer found on terminally sialylated N- as well as O-glycans on a limited number of substrates. Its hydrodynamic, electrostatic, and steric properties confer anti-adhesive characteristics to its carrier proteins. Consequently, polySia modulates signaling downstream of its substrates and impacts protein-protein and cell-cell interactions. It is therefore important in the development of nervous system, learning, memory, regeneration as well as immune function but is also believed to promote tumor cell metastasis. Neuropilin-2 (NRP-2) is a VEGF and semaphorin co-receptor, which has been shown to be exclusively polysialylated on the O-glycans between its MAM domain and FV/VIII-2 domain in mature dendritic cells and microglia.

In the present work, we have characterized the molecular mechanism of NRP-2 polysialylation. Using domain deletion mutants and chimeric proteins, we have demonstrated that NRP-2 follows the 'two-domain paradigm' that our laboratory has extensively studied for Neural Cell Adhesion Molecule (NCAM). The NRP-2 MAM domain serves as a recognition domain by polysialyltransferase (polyST) ST8Sia-IV for polysialylation of O-glycans in the adjacent linker region; however, composition of the linker region may also play a secondary role in NRP-2 polysialylation. Furthermore, a specific acidic patch formed by residues $Glu^{652}$ and $Glu^{653}$ on the surface of the MAM domain is important for its polysialylation. These studies also identified NRP-1 as a potential polyST substrate owing to its recycling to the compartments expressing polySTs, despite its weaker polyST recognition elements.

We then sought to assess whether specific basic residues within ST8Sia-IV polybasic region (PBR) play a role in recognition and polysialylation of NRP-2, as previously shown for NCAM. Quite unexpectedly, mutational analysis and competition experiments aimed at identifying PBR basic residues involved in NRP-2 recognition also delineated PBR basic residues involved potentially in the elongation of polySia on its glycans as well as those involved in maintaining the local structure of the ST8Sia-IV PBR. In order to evaluate the possibility that the PBR mutations could impact the polySia chain elongation, we assessed autopolysialylation of the PBR mutants considering a model of ST8Sia-IV that suggested a role for the polyST basic residues in

anchoring the growing polySia chain. These experiments suggested a relationship between polyST autopolysialylation and NRP-2 polysialylation. Based on our results, we propose a model that suggests a novel role of polyST autopolysialylation in extension of polySia chains onto short O-glycans of NRP-2 wherein the polySia chain on the ST8Sia-IV, competes with the growing polySia chain on NRP-2 to bind to relatively basic N-terminal domain(s) of NRP-2.

### (93) The mysterious case of the human Kupffer cell receptor

Maureen E. Taylor, Tom Snelling and Kurt Drickamer
*Imperial College London*

The Kupffer cell receptor mediates clearance of serum glycoproteins. The receptor was isolated from rat liver and a cDNA was subsequently cloned from this source. Although initially described as a fucose-binding receptor, glycan array analysis has suggested that the primary targets are galactose-terminated glycans. The gene encoding the mouse ortholog has been characterized and the mouse receptor has been linked to clearance of de-sialylated platelets. Previous attempts to clone a human ortholog of the Kupffer cell receptor were unsuccessful. However, annotations of the current version of the human genome include the CLEC4F gene on chromosome 2, which would correspond to the Kupffer cell receptor. In light of revisions to the genome sequence, the possibility that humans express a functional Kupffer cell receptor has been re-examined.

Several alternatively spliced versions of transcripts have been predicted from the CLEC4F gene in primary assembly GRCh38.p7 of the human genome. Each of these transcripts encodes the cytoplasmic N-terminus, transmembrane sequence, and extended coiled-coil sequence present in the rat protein. However, none encodes a full-length C-terminal C-type carbohydrate-recognition domain (CRD). Examination of the gene reveals that there is an adjacent sequence that could form an additional exon that would encode the missing portion of the CRD. However, three mutations prevent expression of a functional sugar-binding receptor: an in-frame stop codon at the end of the preceding intron, a mutation in the splice acceptor site, and a nonsense mutation in a key conserved amino acid residue in the sugar-binding site. PCR analysis of transcripts confirms the absence of a spliced transcript containing the final exon. Truncated transcripts are present at low abundance, but are not liver-specific as in rats and mice.

These results suggest that humans do not express a functional version of the Kupffer cell receptor. Analysis of genomes of other primates indicates that close relatives of humans, including chimpanzees, do encode full-length receptor. Functional protein has also been cloned from more distance species including cow. Interestingly, in all cases the CLEC4F gene is immediately adjacent to the CD207 gene, which encodes langerin. Comparison of both the CRD sequences and the overall organization of the Kupffer cell receptor and langerin indicates that these two proteins, with very different binding specificity, arose from a gene duplication. The apparently specific loss of the human Kupffer cell receptor in humans may be correlated with concurrent human-specific mutations that result in altered glycosylation. Alternatively, inactivation of the gene may be driven by specific pathogens that target this receptor. Absence of the receptor also has implications for targeting of glycoconjugates to cells in human liver.

### (94) Small Molecule Inhibition of the Oligosaccharyltransferase

Natalie Rinis[1], Reid Gilmore[2] and Joseph N. Contessa[1]
[1]*Department of Therapeutic Radiology, Yale University School of Medicine;* [2]*Department of Biochemistry and Molecular Pharmacology, University of Massachusetts*

Introduction: The oligosaccharyltransferase (OST) is a multisubunit enzymatic complex with two catalytic subunits; STT3A or STT3B. NGI-1 is a small molecule OST inhibitor that targets both STT3A and STT3B. We sought to understand NGI-1 structure activity relationships with respect to STT3A and STT3B activities and to develop small molecule inhibitors that exclusively target individual catalytic subunits.

Methods: We developed a functional glycosylation reporter using the luciferase gene (ER-LucT) as well as a second marker of glycosylation using the Halo protein (Halo3N) through addition of N-linked glycosylation consensus sequons. These proteins were used to measure inhibition of glycosylation in 293 cells with or without CRISPR-Cas9 knockout of STT3A or STT3B, respectively. 19 chemical analogs were synthesized and evaluated by western blot or luciferase assay to determine structure activity relationships using a numerical grading system.

Results: Small molecule inhibition of ER-LucT glycosylation enhances luminescence in parental and STT3B knockout 293 cells. The reporter is not efficiently glycosylated in the absence of STT3A. In contrast, efficient glycosylation of the Halo-3N protein measured by Western Blot is observed in both STT3A and STT3B KO cells. Stable Halo3N expression in each cell type thus provides a marker for either STT3A or STT3B catalytic activity. Halo3N has a C-terminal NXT site strictly dependent on STT3B and two N-terminal sites that were found to be modified by both catalytic subunits. NGI-1 blocks OST activity with either STT3A or STT3B knockout confirming targeted inhibition of both OST complex types. The effects of NGI-1 analogs were screened in Halo3N expressing cells using a final concentration of 10uM to determine specificity for OST complexes containing either STT3A or STT3B. Derivatives of the molecule's functional groups changed the catalytic subunit specificity. In general, larger substitutions for the pyrrolidine or sulfonamide moieties significantly or completely eroded potency for STT3A inhibition. Substitution of the aminothiazole group was tolerated, but also demonstrated reduced potency for inhibiting both STT3A and STT3B, and was pivotal for STT3A targeting. Data generated with the Halo-3N protein were in line with that from ER-LucT in STT3B KO cells. Results from these model systems identify an analog, NGI-19, that is an STT3B

specific inhibitor although with reduced potency compared to the parent compound. The B-specificity of NGI-19 was confirmed with endogenous glycoproteins such as EGFR, MET, and IGF1Rβ in the absence of STT3A.

Conclusions: NGI-1 blocks activity of OST complexes containing either the STT3A or STT3B catalytic subunit. Here we introduce NGI-19 that is a chemical analog of NGI-1 and is a STT3B specific small molecule inhibitor.

### (95) Oligosaccharyltransferase Inhibition Enhances Glioma Radiosensitivity

Marta Baro and Joseph N. Contessa
*Department of Therapeutic Radiology, Yale University School of Medicine*

PURPOSE: Receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), have been validated as therapeutic targets to enhance radiation sensitivity. However, parallel RTK signaling reduces the effects of EGFR inhibition in glioma and therefore new targets for blocking receptor driven survival signaling are needed.N-linked glycosylation is an endoplasmic reticulum (ER) co- and post-translational modification common to RTKs that is crucial for receptor activation. We hypothesized that partial inhibition of N-linked glycosylation with NGI-1 could be a new therapeutic approach to disrupt parallel RTK signaling in order to radiosensitize malignant glioma.

EXPERIMENTAL PROCEDURES: RTK activity and dependent downstream signaling were assessed by phosphoblot analysis. Radiosensitivity was determined through clonogenic survival analysis. Cell proliferation was assessed with MTT assays over 5 days of drug treatment, cell cycle analysis was performed by flow cytometry and DNA damage was assessed by H2AX immunoflorescence. We generated a model for glycosylation-independent RTK signaling by fusing the extracellular domain of CD8 (which contains no N-linked sequons) with the intracellular domain of EGFR.Cells with stable CD8-EGFR expression were compared to parental cells in order to investigate the mechanism of NGI-1 effects on radiosensitivity.

RESULTS: Glioma cell lines have distinct patterns of RTK (ErbB family, PDGFR, MET and FGFR1) expression and activation that contribute to proliferation and survival signaling.NGI-1 was shown to reduce the glycosylation, protein levels, and activation for each receptor. Cell lines with significant ErbB family RTK activation were radiosensitized by NGI-1 while cell lines without activation of this RTK family were not radiosensitized by NGI-1. NGI-1 also enhanced the effects of cytotoxic chemotherapy in the malignant glioma cells with high levels of ErbB family RTK signaling. The blockade of ErbB family RTK signaling with NGI-1 caused a significant G1 cell cycle arrest compared to controls, and resulted in an increase in DNA damage after radiation exposure as shown by the accumulation of H2AX foci formation. Using glycosylation-independent CD8-EGFR expressing cells with constitutive EGFR kinase activity, we found that the effects of NGI-1 on G1 arrest, DNA damage, and cellular radiosensitivity were reversed. Comparison of the CD8-EGFR and parental cells also showed that NGI-1 blocks radiation-induced activation of AKT in parental but not in the radioresistant CD8-EGFR expressing cells. This result is similar to the effect of NGI-1 on glioma cells without ErbB RTK family signaling, where NGI-1 does not block AKT activation.

CONCLUSION: This study suggests that the inhibition of N-glycosylation with NGI-1 is a novel approach to radiosensitize RTK-dependent glioblastoma tumors. Our data supports further basic and pre-clinical investigations with NGI-1 as a potential cancer therapy

### (96) Fluorescent Imaging of N-linked Glycosylation and Oligosaccharyltransferase Activity

Wei Cui and Joseph N. Contessa
*Department of Therapeutic Radiology, Yale University School of Medicine*

**Purpose:** N-linked glycosylation is the most frequent protein modification of the secretory pathway and regulates protein folding, localization, and function. Quantitation of N-linked glycan site occupancy has been achieved with luciferase-based reporter systems, however, this approach does not provide the ability to detect cellular heterogeneity or to select populations with altered N-linked glycosylation. We therefore developed a fluorescent-based reporter system to dynamically monitor glycosylation in living cells and to implement this model for genetic screening approaches.

**Methods:** Using the bacterial halogenase (Halo) protein, we generated constructs with an in-frame endoplasmic reticulum (ER) translation leader sequence from the EGFR along with point mutations that introduced new NXS/T consensus sequons for N-linked glycosylation. Non-ER translated constructs were used as controls to insure that mutations did not affect Halo-ligand interactions. Glycosylation of each site was evaluated by size shifts on western blot. A 492/520 excitation/emission Oregon green Halo ligand was used to quantify fluorescence in 96 well plate format and fluorescent cells were also viewed with Hoechst counterstaining of the nuclei using a 20X objective lens. An A549 human adenocarcinoma cell line with stable transfection of Halo-1N was generated with G418 selection. Flow cytometry was used to determine the change in signal intensity and parameters for single cell sorting.

**Results:** Introduction of new glycosylation sites into the ER-Halo reporters increased the protein's molecular weight consistent with N-linked glycosylation. We identified the ER-Halo-1N as a sensitive reporter construct where changes in protein glycosylation directly correspond to increased measurements of fluorescence. Introduction of multiple sequons did not improve the sensitivity of the ER-Halo. Tunicamycin (1 μM, an inhibitor of DPAGT1) and NGI-1 (10 μM, and oligosaccharyltransferase inhibitor) induced an

88% and 52% increase in fluorescence, respectively. Moreover the Halo-1N displayed low background activity and dramatically increased the overall number of fluorescent cells, with virtually all cells displaying ER fluorescence. These values were also positively correlated with increased dose or treatment time. Wash out experiments removing NGI-1 demonstrated a time dependent loss of fluorescence showing that glycosylation could be dynamically monitored. Flow cytometry experiments with combinations of NGI-1 treated and untreated cells showed that >99% of treated cells could be sorted from this mixed population.

**Conclusions:** Our work introduces a new technique for fluorescently monitoring the status of N-linked glycosylation and OST activity in living cells. The ER-Halo provides the ability to quantify and select individual cells with aberrant N-linked glycosylation and thus has potential applications for evaluating genetic factors that modify this protein modification.

### (97) CRISPR-Cas9 Dissection of Heparan Sulfate

Ryan J. Weiss[1], Philipp N. Spahn[2], Nathan E. Lewis[2,3] and Jeffrey D. Esko[1,3]

*[1]Department of Cellular and Molecular Medicine, University of California, San Diego, San Diego, CA; [2]Department of Pediatrics, School of Medicine, University of California, San Diego, San Diego, CA; [3]Glycobiology Research and Training Center, University of California, San Diego, San Diego, CA*

Heparan sulfate proteoglycans (HSPGs) are expressed on virtually all animal cells and in the extracellular matrix. Each HSPG consists of a core protein with one or more covalently attached linear heparan sulfate (HS) chains composed of alternating glucosamine and uronic acids that are heterogeneously *N*- and *O*-sulfated. These complex cell surface carbohydrates regulate important biological processes including cell proliferation and development through their interaction with a large number of matrix proteins and growth factors. The arrangement and orientation of the sulfated sugar residues of HS specify the location of distinct ligand binding sites on the cell surface, and these modifications can vary temporally during development and spatially across tissues. While most of the enzymes involved in HS biosynthesis have been studied extensively, much less information exists regarding the specific mechanisms that give rise to the variable composition and binding properties of HS. A genome-wide CRISPR/Cas9-mediated screen was developed to uncover and to characterize novel genes other than those encoding known HS biosynthetic enzymes. A lentiviral single guide RNA (sgRNA) library was utilized to knock down gene expression across the entire genome in a human malignant melanoma cell line. Subsequently, a high-throughput screening assay was adapted to identify lentiviral-encoded sgRNAs that induce resistance to cytotoxins whose action depends on HSPGs. Parallel screens using alternative HS-dependent toxins, flow cytometry, or plant lectins that cause cytotoxicity dependent on other types of glycosylation were performed in order to sort genes that selectively affect HS biosynthesis. We identified previously studied genes essential for HS formation and factors involved in the intoxication of cells. Furthermore, we uncovered potential candidate genes whose function is unknown relative to HS biosynthesis. Top hits from the screens were characterized and categorized based on their predicted gene functions and are currently being individually validated and examined for their potential involvement in the regulation of HS biosynthesis. Overall, these studies will give us a better understanding of the genetic regulatory factors involved in HS biogenesis and define targets for drug development.

### (98) Novel glyco-regulation of SCF Ubiquitin ligases is a potential drug target for control of Toxoplasma

Christopher M. West[1,2], Msano Mandalasi[1,2], Osman Sheikh[3], David Thieker[3], John Glushka[3], Tongri Liu[4], Agnieszka Lis[5], Christopher J. Schofield[4] and Ira J. Blader[5]

*[1]Dept. of Biochemistry & Molecular Biology, University of Georgia, Athens, GA USA 30602; [2]Center for Tropical and Emerging Global Diseases, University of Georgia; [3]Complex Carbohydrate Research Center, University of Georgia; [4]Dept. of Chemistry, Oxford University, Oxford, UK; [5]Dept. of Microbiology & Immunology, University at Buffalo, Buffalo, NY*

SCF (Skp1/Cullin-1/F-box) polyubiquitin ligases contribute importantly to regulation of the proteome of all eukaryotes including the apicomplexan *Toxoplasma gondii*, the agent of human toxoplasmosis. The adaptor subunit Skp1 of *Toxoplasma* and other protists, including the model organism *Dictyostelium* (a social amoeba), is conditionally modified by an $O_2$-dependent prolyl 4-hydroxylase (PhyA) and five sugar nucleotide-dependent glycosyltransferases, resulting in the assembly of a discrete pentasaccharide at Pro154. PhyA, evolutionarily related to the HIFα-dependent $O_2$-sensor in humans, is required for optimal plaque formation in fibroblast monolayers and inhibited by compounds under development for treatment of anemias in humans. Our recent identification, disruption and complementation of the five glycosyltransferase genes required to assemble the pentasaccharide glycan reveal that they are also important for growth on fibroblasts. Biochemical and structural studies show that the glycosyltransferases have striking selectivity toward Skp1 *in vitro*, and solely modify Skp1 in biochemical complementation tests. Thus, this remarkably complex 6-gene modification pathway may be dedicated to Skp1 alone in cells. Recent NMR studies have established the linkages of the glycosyltransferase products and showed that, despite differences relative to the *Dictyostelium* Skp1 glycan and the enzymes that assemble it, molecular dynamics studies suggest that effects on Skp1 conformation will be similar. Glycosylation affects interaction of Skp1 with F-box proteins in both *Dictyostelium* and *Toxoplasma*, which is likely explained by action of the glycan on Skp1 in *cis*. Overall, our studies suggest a new, potentially druggable, paradigm

of environmental and metabolic regulation of *Toxoplasma* that is mediated via the turnover of selected protein.

### (99) Making Glycoproteomics via Mass Spectrometry More Accessible to the greater Scientific Community

Marc D. Driessen[1], Catherine C. Going[2,3], Chistina M. Woo[4], Sharon J. Pitteri[2,3] and Carolyn R. Bertozzi[1,3]
[1]*Department of Chemistry, Stanford University;* [2]*Department of Radiology, Stanford School of Medicine;* [3]*Howard Hughes Medical Institute;* [4]*Department of Chemistry, Harvard University*

We have recently demonstrated a method that, for the first time, is capable of facilitating both, glycan structure and attachment site analysis for both N- and O- glycans alike. This method named '*Isotope Targeted Glycoproteomics*' (IsoTaG) allows us to enrich glycopeptides and yields an unprecedented detection of low abundant glycoproteins. All this is achieved without the need to truncate glycan structures, thereby keeping the glycomic information intact. Furthermore the need for complex fractionation protocols is abated, which goes along with a reduction in both the mass spectrometry analysis and computational analysis time. The IsoTag workflow consists of four main steps: 1) Tagging of glycoproteins *via* metabolic labelling (using either azido or alkyne sugars); 2) Introduction of the IsoTag via 'click-chemistry'; 3) Capture of tagged glycoproteins on a solid phase support; 4) (tryptic) digest of captured proteins followed by separate collection of peptides and glycopeptides making use of an acid labile cleavage site incorporated in the IsoTag probe.

Since we have demonstrated the usefulness of the method on our own samples, we have begun to transfer it to other interested laboratories, some of which have no prior glycoproteomic analysis. We aim to make this method a widespread tool for both glycoproteimics experts and non-experts supplying them with a novel approach to generate meaningful glycoproteomic datasets. To this end we plan to further simplify the approach, provide a source of the IsoTag probe, as well as provide interested labs with a set of protocols and standards that can be used as a comparison to adjust and fine tune instrument parameters. This information will be available to the broad community in an online repository (www.IsoStamp.org).

We have also taken the first steps to start a tired evaluation and improvement of the IsoTag workflow and MS conditions by starting an interlaboratory comparison of identical samples. We will use data gathered from a small round-robin test to produce and test standard operating procedures (sample preparation, IsoTag incorporation, MS analysis). These will then be tested for general applicability by disseminating them along with a set of standards to a larger group of laboratories, with a focus on MS analysis (MS core facilities). The results will be integrated, analyzed and used for further improvements.

In parallel we are demonstrating new applications of the IsoTag method by generating biologically relevant data. At the same time we are steadily developing new IsoTag probe systems for a broader range of applications.

### (100) gID: A new strategy for identification of glycan branching patterns using multistage mass spectrometry

Shiwei Sun[1], Chuncui Huang[2], Jingwei Zhang[1], Yaojun Wang[1], Dongbo Bu[1], Yan Li[2] and Wengang Chai[3]
[1]*Institute of Computing Technology Chinese Academy of Sciences.;* [2]*Institute of Biophysics, Chinese Academy of Sciences;* [3]*Imperical College London, London, United Kingdom.;* [4]*University of Chinese Academy of Sciences, Beijing, China*

Glycans play important roles in a variety of biological processes. Their activities are closely related to the fine details of their structures. Unlike the simple linear chains of proteins, branching is a unique feature of glycan structures, making their identification extremely challenging. $MS^2$ is not always sufficient and multiple-stage mass spectrometry ($MS^n$, $n > 2$) is frequently required to provide detailed structural information of glycans and has become the primary method for glycan structural identification. The major difficulty for $MS^n$ is the how to merge the information in all the spectra produced by analysis of one sample to complete the identification. In this work, we propose a new strategy to analysis $MS^n$ spectra. We applied the strategy to assignment of a variety of *N*-glycans and secreted oligosaccharides and to *N*-glycan profiling.

The ability to identify glycan branching structures has been clearly demonstrated with 28 glycan standards including *N*-glycans of the high-mannose, complex and hybrid types, and human milk-related oligosaccharides, within a maximum of four stages of $MS^n$ experiments. For *N*-glycan profiling, GIPS can be used as an additional dimension to the conventional profiling method by assigning individual glycan components as shown in the human serum sample.

Our work on glycan structural analysis together with recent methodological development in various aspects of glycomics, e.g. novel chemical method for the release of *O*- and *N*-glycans and *N*-glycosylation site analysis, will undoubtedly contribute to a better understanding of glycan structure and function in health and disease.

### (101) Amplification and Preparation of Cellular O-glycans using Cellular O-glycome Reporter/Amplification (CORA)

Zhonghua Li[1], Qing Zhang[2], Tatiana Chernova[1], George Wang[2], Xuezheng Song[1], David F. Smith[1], Cummings D. Richard[3] and Tongzhong Ju[4,1]
[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA;* [2]*Department of Chemistry, Georgia State University, Atlanta, GA;* [3]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA;* [4]*Office of Biotechnology Products, Center for*

*Drug Evaluation and Research, Food & Drug Administration, Silver Spring, MD*

Cellular O-glycome Reporter/Amplification (CORA) is a novel, sensitive and versatile method used to profile and amplify mucin type O-glycans from living cells. CORA relies on the uptake of peracetylated benzyl-α-N-acetylgalactosamine [Bn-α-GalNAc(AC)3] by cells, which results in its deacetylation to generate Bn-α-GalNAc. This is transported into the Golgi apparatus where Bn-α-GalNAc mimics the O-glycan precursor GalNAc-α1-O-Ser/Thr (Tn antigen) and is modified and elongated by different glycosyltransferases to generate the Bn-O-glycans. The Bn-O-glycans, which reflect the O-glycome of the cell being studied, are then secreted into the media. These Bn-O-glycans can easily be isolated by reverse-phase chromatography, permethylated and profiled by MALDI-TOF MS and further characterized by MSn. Although the benzyl group of the aglycone provides a tag to monitor the separation and quantify individual glycans, the UV signal is not sufficiently sensitive to detect the low abundance O-glycans, and it is difficult to chemically modify or install functional groups that could be used to print onto the glass slide for glycan microarray or further modify for functional glycomic analyses. To address this limitation of CORA and determine if this method can be used for preparing tagged glycan libraries of cellular O-glycomes, we have explored a variety of different aglycones of peracetylated aGalNAc. One of the compounds tested was 4-Azido-Bn-α-GalNAc$(Ac)_3$, which we have shown in equivalent to Bn-α-GalNAc$(Ac)_3$ in being taken up and processed by all of the cell lines tested to report/amplify a series of azido-Bn-O-glycans. The azido functional group of 4-Azido-Bn-α-GalNAc can be chemically conjugated with different probes/tags. Importantly, these azido-Bn-O-glycans can be efficiently conjugated with a fluorescent tag containing a free amino group. The fluorescent signal provides a sensitive method for monitoring the separation during HPLC, so that the low abundance O-glycans can be easily purified and free amino group can be utilized for printing O-glycans on the NHS-glass slides to produce glycan microarrays representing the O-glycome of cultured cell lines. This approach will be used to determine the feasibility of preparing cellular O-glycans using cells as O-glycan factories to facilitate both cellular O-glycomics and functional O-glycomics studies. [This work was supported by NIH Grant U01CA207821 to TJ].

### (102) A Yeast Model to Define the Molecular Interactions between Core 1 b3GalT and its Molecular Chaperone Cosmc

Tatiana A. Chernova[1], Qian Wang[1,3], Yuliang Jiang[1,3], David F. Smith[1] and Tongzhong Ju[2,1,3]

[1]*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA;* [2]*Office of Biotechnology Products, Food & Drug Administration, Silver Spring, MD;* [3]*Department of Oncology, the Capital Medical University, Beijing Chaoyang Hospital, Beijing, China*

O-glycosylation is one of most common protein posttranslational modifications. O-glycans on glycoproteins play pivotal roles in many biological process, including cell signaling, cell adhesion, and immune modulation. Not surprisingly, in pathological situations such as cancer, O-glycans on diseased cells are altered, and more importantly the aberrant O-glycans could contribute to the development, progression and metastasis of tumor. O-glycans are synthesized by sequential actions of glycosyltransferases. Among them, Core 1 3Galactosyltransferase (core 1 b3GalT, T-synthase) normally residing in the Golgi apparatus is the key enzyme in the O-glycosylation pathway responsible for converting the Tn antigen to core-1 structure, which is the precursor of most O-glycans on cell surface. Interestingly, biosynthesis of active Core 1 b3GalT requires Cosmc (Core 1 b3GalT Specific Molecular Chaperone), which mainly localizes in endoplasmic reticulum (ER), and assists the folding of Core 1 b3GalT by preventing its aggregation/proteasomal degradation. Alterations in *Cosmc* are associated with human diseases, such as Tn-syndrome, and cancers. However, the molecular interactions between Core 1 b3GalT and Cosmc are not well understood. We are developing a yeast model to investigate the novel interactions of Cosmc with Core 1 b3GalT. For this purpose we constructed plasmids with different selection markers expressing T-synthase and Cosmc under control of copper inducible promoter. Plasmids were transformed into the yeast *Saccharomyces cerevisiae* and expression of T-synthase and Cosmc was confirmed by Western blot analysis using protein specific antibody. We used 4-methylumbelliferone fluorescence assay to measure Core 1 b3GalT activity in yeast cells crude extract and demonstrated that similar to mammalian cells T-synthase activity requires presence of its chaperone Cosmc. To study protein localization in the yeast cell we used plasmids expressing T-synthase and Cosmc fused with Green and mCherry Fluorescent Protein correspondingly. We used fluorescent microscopy to image proteins and demonstrated that in yeast living cells, like in mammalian cells, T-synthase and Cosmc co-localized in the ER. It has been shown previously in mammalian cells that T-synthase aggregates when Cosmc is not present or mutated. We found that T-synthase in yeast cells also forms aggregates. To characterize these aggregates we used Semi-Denaturating Detergent Agarose Gel Electrophoresis, a method for detecting and characterizing large protein polymers. Our results suggest that Core 1 b3GalT protein forms aggregates with amyloid–like features. We are investigating how Cosmc interacts with Core 1 b3GalT and possibly other proteins/chaperones to understand how Cosmc assists the folding of Core 1 b3GalT and how dysfunction of Cosmc may cause human disease. [This work was supported by NIH Grant U01CA207821 to TJ].

### (103) Quantitative O-Glycomics: metabolic Stable Isotopic Labeling of O-glycomes of cultured Cells (SILOC)

Tatiana A. Chernova[1], Zhonghua Li[1], Yuliang Jiang[1,3], Qian Wang[1,3], David F. Smith[1] and Tongzhong Ju[2,1,3]
*[1]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA; [2]Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, MD; [3]Department of Oncology, Affiliated Beijing Chaoyang Hospital, The Capital Medical University, Beijing, China*

Glycans linked to Asparagine (N-glycans) and Serine/Threonine (O-glycans) play critical roles in the molecular interactions that govern many important biological systems. Furthermore, alterations of glycans which are often associated with diseases can significantly affect cellular properties and functions. To fully understand the biological roles of glycans known as functional glycomics, information on cellular glycans (glycomes) is necessary. Glycan analysis, however, often requires release from glycoconjugates prior to analysis by Mass Spectrometry (MS). While N-glycan analysis is feasible, O-glycan analysis is challenging since no endoglycosidase is available. To address this limitation, we recently developed a novel technology termed *Cell O-glycome Reporter/Amplification* (CORA), which is a simple, sensitive, and versatile method for profiling O-glycans of cultured cells fed with low concentrations of peracetylated Benzyl-α-N-acetylgalactosamine [Bn-α-GalNAc$(Ac)_3$]. The products, Bn-O-Glycans synthesized by cells are secreted into the media and easily isolated for analysis by MS. Here, we advanced the CORA technology to a Quantitative-CORA by "metabolic Stable Isotopic Labeling of O-glycomes of cultured Cells (SILOC)" with [$^{13}C_6$]Bn-α-GalNAc$(Ac)_3$. Any two types of cells or the same cell line cultured at different conditions were metabolically labeled with {[$^{12}C_6$]Bn-α-GalNAc$(Ac)_3$} (Light)and [$^{13}C_6$]Bn-α-GalNAc$(Ac)_3$ (Heavy) respectively. Importantly, [$^{13}C_6$]Bn-α-GalNAc$(Ac)_3$ was unbiasedly uptaken and modified to report cellular O-glycans as[$^{12}C_6$]Bn-α-GalNAc$(Ac)_3$. The mixture of Light and Heavy media from different cells was then processed and finally analyzed by MS. The [$^{12}C_6$]- and [$^{13}C_6$]Bn-O-glycans with 6-Da differences in Mass were easily compared. We have tested the method on a human lung adenocarcinoma A549 cells cultured on a regular surface and Matrigel Matrix and other cell lines with or without a genetic manipulation; the Qualitative and Quantitative differences of O-glycans between those culture conditions and between cell lines were readily resolved. For example, while sialyl-Core 2 structure from A549 cells cultured at two different conditions did not change, the fucosylated and sialylated complex Core-2 O-glycans, as well as some novel, unidentified structures decreased in A549 cells on the Matrigel. This SILOC-Quantitative CORA technology for Quantitative O-glycomics will facilitate the study of functional O-glycomics and the discovery of new glyco-biomarkers for human diseases such as cancer. [This work was supported by NIH Grant U01CA207821 to TJ].

### (104) Homogeneous Bioluminescent Nucleotide Detection Assays for Glycosyltransferases and Other PTM Enzymes

Hicham Zegzouti, Laurie Engel, Gediminas Vidugiris, Juliano Alves, Kevin Hsiao and Said Goueli
*Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711 USA*

Post translational modifications (PTMs) are central to all aspects of biological regulation. PTMs amplify the diverse functions of the proteome by covalently adding functional groups to proteins. These modifications include phosphorylation, methylation, acetylation, glycosylation, ubiquitination, and influence many aspects of normal cell biology and pathogenesis. Examples of post translational modification enzymes include but not limited to, Kinases/Phosphatases, Methyltransferases/ Demethylases, Acetyltransferases/Deacetylases, and Glycosyltransferases/Glucanases. Under normal physiological conditions, the regulation of PTM enzymes is tightly regulated. However, under pathological conditions, these enzymes activity can be dysregulated, and the disruption of the intracellular networks governed by these enzymes leads to an array of diseases including cancer and inflammation. Consequently, PTM enzymes have become important targets for drug discovery creating a need for development of activity detection assays. Although several technologies were developed to assess the activity of these targets, most suffer from limitations that hamper their application in high throughput drug screening. Many PTM enzymes utilize nucleotides as substrates or generate them as reaction products. Each nucleotide is a common product of a large group of enzymatic reactions. Thus, the development of detection assays that monitor nucleotide production with high performance will amplify the number of PTM enzymes that could be studied and will have significant impact on diverse areas of research. This presentation will showcase how cellular mechanisms driven by PTM can be addressed by analyzing the biochemical activities of the involved enzymes using a suite of bioluminescent nucleotide detection assays. In particular, we will discuss the development of four bioluminescent assays for measuring Glycosyltransferase activities based on UDP, GDP, UMP and CMP detection. Each assay is performed in a single-step detection that relies on converting simultaneously the nucleotide product to ATP, followed by conversion to light in a luciferase reaction. These GT assays are highly sensitive and robust. We will present example applications of these assays including studies on specificity of transfer of different sugars to different acceptors. We will show their use in screening for specific glycosyltransferase inhibitors as well as in studies of their modes of action. Development of these nucleotide detection assays will enable the investigation of a large number of GTs, which may have significant impact on diverse areas of Glycobiology research.

### (105) Glycans marking subpopulations of pancreatic cancers: characterizing structures derived from type-II N-acetyl-lactosamine using on-chip analysis

Peter Y. Hsueh[1,2], Zachary Klamer[1], Randall E. Brand[3] and Brian B. Haab[1]

*[1]Center for Cancer and Cell Biology, Van Andel Research Institute, Grand Rapids, MI; [2]Cellular and Molecular Biology, Michigan State University, East Lansing, MI; [3]University of Pittsburgh Medical Center, Pittsburgh, PA*

The current best biomarker for pancreatic cancer, the CA19-9 glycan, is elevated in the blood of about 75% of patients at a cutoff that gives a 25% false positive rate, but no biomarkers are available to assess patients for whom CA19-9 is not useful. We recently found that glycans related to the CA19-9 antigen are complementary biomarkers to CA19-9. Characterizing the nature of the new biomarkers would help to optimize detection and understand the biochemistry of the subtypes, but we did not have an effective method for characterizing the glycans, mainly owing to the small amounts of clinical specimens that were available. We recently developed a protocol and analysis method for probing glycan structures in small amounts of material, a technique called on-chip glycan modification and probing (on-chip gmap). Here we used on-chip gmap to test the hypothesis that structures derived from type-2 N-acetyl-lactosamine (LacNAc) are biomarkers for subpopulations of pancreatic cancers, in contrast to the CA19-9 glycan, which is derived from type-1 LacNAc. We captured glycoproteins directly from clinical specimens onto low-volume antibody microarrays (2.2 × 2.2 mm), treated parallel arrays with three different exoglycosidases (α2-3 neuraminidase, α1-3,4 fucosidase, and β1-4 galactosidase) to expose underlying features, and probed the native and exposed features with a panel of lectins or glycan-binding antibodies. We employed in-house software called MotifPredict to interpret the raw data and provide predictions about the glycan motifs that are present in each plasma sample. We found evidence that glycoforms of the proteins MUC5AC and MUC16 displaying sulfated sialyl-Lewis X(s) are elevated in many patients who do not elevated CA19-9, and that glycoforms displaying non-fucosylated sialyl-LacNAc mark another subset of patients. The majority of samples that were low in CA19-9 showed elevated staining by the recombinant protein Siglec-F, and the combined use of the cancer-associated glycoforms provided added value in biomarker performance. The results show that at least two different glycans derived from type-2 LacNAc, in addition to sLeX, are complementary biomarkers for pancreatic cancers, and that on-chip gmap is useful for finding disease-associated features using clinical specimens.

### (106) Biochemical Characterization of Functional Domains of the Chaperone Cosmc

Melinda S. Hanes[1,2], Kelley W. Moremen[3] and Richard D. Cummings[1,2]

*[1]Beth Israel Deaconess Medical Center; [2]Harvard Medical School; [3]Complex Carbohydrate Research Center, University of Georgia*

Cosmc is an endoplasmic reticulum chaperone essential for normal protein O-GalNAc glycosylation through regulation of T-synthase, its single client. Loss-of-function of Cosmc results in Tn antigen expression, which is associated with multiple human diseases including cancer. Despite intense interest in the Tn antigen, the structure and function of Cosmc, including domain organization, secondary structure, oligomerization, and co-factors are unknown. Limited proteolysis experiments show that Cosmc contains a structured N-terminal domain (Cosmc residues 29-256), and biochemical characterization of the corresponding truncated protein,Cosmc256*,indicates wild type chaperone activity. Interestingly, CosmcE152K, which displays loss of function *in vivo*, exhibits wild type-like activity *in vitro*. Cosmc and CosmcE152K heterogeneously oligomerize and form monomeric, dimeric, trimeric, and tetrameric species, while Cosmc256*is primarily monomeric as characterized by chemical crosslinking and blue native page electrophoresis. Additionally, Cosmc binds selective divalent cations in thermal shift assays and metal binding is abrogated by the Cosmc256*, and altered by the E152K mutation. Therefore, the N-terminal domain of Cosmc is responsible for T-synthase binding and chaperone function, whereas the C-terminal domain is necessary for oligomerization and metal binding. Our results provide new structure-function insight to Cosmc, indicate that Cosmc behaves as a modular protein and suggests points of modulation or regulation of *in vivo* chaperone function.

### (107) Mutations in ATP6AP2 are associated to Congenital Disorders of Glycosylation with autophagic defects

Romain Péanne[1], Magda Cannata Serio [2,5], Maria A. Rujano[2,5], Ganna Panasyuk[3,4,5], Janine Reunert[6], Dulce Quelhas[7], Michael Schwake[8], Sandrine Duvet[9], Francois Foulquier[9], Gert Matthijs[1], Thorsten Marquardt[6] and Matias Simons[2,5]

*[1]University of Leuven (KU Leuven), Department of Human Genetics, Leuven, Belgium; [2]Laboratory of Epithelial Biology and Disease, Imagine Institute, Paris, France; [3]Institut Necker-Enfants Malades (INEM), Paris, France; [4]INSERM U1151/CNRS UMR 8253, Paris, France; [5]Université Paris Descartes, Sorbonne Paris Cité, Paris, France; [6]Universitätsklinikum Münster, Klinik für Kinder - und Jugendmedizin, Münster, Germany; [7]Biochemical Genetics Unit, Centro de Genetica Medica Doutor Jacinto Magalhães, CGMJM, Centro Hospitalar do Porto, Abel Salazar Institute of Biomedical Sciences University of Porto, Porto, Portugal; [8]Northwestern University, Feinberg School of Medicine, Chicago, IL, USA; [9]University of Lille, CNRS, UMR 8576, Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France*

The V-ATPase is a multi-subunit proton pumpconsistingof a V1 domain responsible for ATP hydrolysis and a V0 domain responsible for proton translocation, and therefore organelle acidification. Organelle acidification has been shown to mediate different intracellular processes such as protein sorting, vesicular trafficking, receptor-mediated

endocytosis, and lysosomal/autophagosomal protein degradation.The assembly of the pump commences in the endoplasmic reticulum with the formation of the proton pore V0 under the control of assembly factors. Recently, it has been shown that mutations in V-ATPase subunits and assembly factors cause Congenital Disorders of Glycosylation (CDG). By using whole exome sequencing in a cohort of unexplained CDG cases, we identify three individuals from two unrelated families with point mutations in the X-linked gene of the accessory V-ATPase subunit ATP6AP2. All the patients presented a similar clinical phenotype with glycosylation defects, liver disease, cutis laxa, psychomotor impairment and immunodeficiency. Both point mutations reside in the extracellular domain and affect the protein stability, processing, interaction with V0 assembly factors and, consequently, V-ATPase activity. The conditional liver inactivation of ATP6AP2 in a murine model revealed impaired serum protein glycosylation and autophagic flux. Finally, the introduction of the mutation into Drosophila led to reduced survival, impaired protein stability and defects in lipid metabolism and autophagy as a result of impaired V-ATPase activity. Overall, we show that ATP6AP2 deficiency belongs to a new subgroup of CDG, featured by V-ATPase and autophagy defects, and define a new role for ATP6AP2 in V0 assembly in the endoplasmic reticulum.

### (108) The odyssey of MAGT1: from magnesium channel back to N-glycosylation?

Eline Blommaert[1], Romain Péanne[1], Christophe Verstegen[1], Valérie Race[1], Erika Souche[1], Liesbeth Keldermans[1], Daisy Rymen[2], Jaak Jaeken[2] and Gert Matthijs[1]
*[1]Laboratory for Molecular Diagnosis, Centre for Human Genetics, KU Leuven, Belgium; [2]Centre for Metabolic Diseases, University Hospital Leuven, Belgium*

Since Congenital Disorders of Glycosylation (CDG) are a genetically heterogeneous group of metabolic disorders, we have established a Custom Capture Assay. The panel included 79 genes: known CDG genes as well as candidate genes. With this approach two patients with hemizygous mutations in the candidate gene *MAGT1* were identified. A missense mutation was found in an 11-year-old boy (P1), and a *de novo* nonsense mutation was identified in a 9-year-old boy (P2). Both children displayed similar clinical features with intellectual disability, developmental delay and macrocephaly.

Mutations in *MAGT1* have previously been described to cause an X-linked immunodeficiency disease (XMEN), characterised by chronic EBV infections. These studies claim that MAGT1 is a $Mg^{2+}$ transporter located to the plasma membrane (PM) and required for $Mg^{2+}$ uptake in vertebrate cells[1]. However, this contradicts reports showing that MAGT1 is localised in the ER and a subunit of the oligosaccharyltransferase (OST) complex[2]. The latter plays a very important role in N-glycosylation since it catalyses the transfer of the preassembled oligosaccharide precursor to nascent proteins. The two identified patients offer us the opportunity to confirm the causal role of the gene in CDG and to study the function of MAGT1 in glycobiology.

The hypothesis is that **MAGT1 is a component of the OST complex, and is involved in the glycosylation of a subset of N-glycoproteins. One of these substrates could be a $Mg^{2+}$ transporter**, which would link the defect to the observations in XMEN patients.

The expression levels of the different subunits were investigated by transcript analysis. A reduction of more than 60% in *MAGT1* expression was observed in the patient presenting the stop mutation. Interestingly, *TUSC3* (the homologue of *MAGT1*) transcript level was upregulated in the patients' cells (2 to 3-fold). Furthermore, the expression of the catalytic subunits of the OST complex was altered in P2. These results point towards a rescue mechanism in the patients. Moreover, a study of MAGT1 dependent substrates by western blot confirms that these proteins are hypoglycosylated in the patients' cells. Furthermore, to elucidate the localisation of MAGT1 we have conducted cell surface biotinylation and confocal microscopy, which showed that the protein is localised both at the ER ánd PM. By transfecting HEK293T MAGT1$^{-/-}$ cells with the different transcripts of the gene, it was shown that these transcripts have a different localisations. The multiple isoforms of MAGT1 will be assessed soon for their oxidoreductase activity and electrophysiological properties, which will allow to determine if MAGT1 is a **moonlighting protein** performing the two previously described functions or not.

In conclusion, we identified the first MAGT1-CDG patients and were able to confirm the role of the protein in N-glycosylation.

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### (109) Role of protein glycosylation in Drosophila heart physiology and development

Brooke Howell, Ishita Chandel and Vladislav Panin
*Department of Biochemistry and Biophysics Texas A&M University, College Station, Texas 77843*

Previous studies of the mammalian heart have uncovered protein glycosylation as a necessary component in the regulation of cardiac function. Alterations in these post-translational modifications are detrimental to cardiac physiology, as they have been shown to affect voltage-gated ion channels, thereby resulting in arrhythmias and cardiomyopathies. The molecular and genetic mechanisms behind these cardiac phenotypes still remain poorly understood. We aim to uncover pathological mechanisms underlying heart phenotypes using the *Drosophila* heart as a model. The *Drosophila* heart has served as a powerful tool to study several cardiac genes with significant and directly translatable roles in human heart disease. This model provides a low-cost *in vivo* system that is amenable to high-throughput

screening. In addition, the *Drosophila* model provides key advantages for studying sialylation, as *Drosophila* has substantially decreased genetic redundancy and complexity of neural glycosylation. Employing this system, we have analyzed knockout mutants for the gene encoding the final enzyme in the sialylation pathway responsible for the transfer of sialic acid residues to glycoproteins, *Drosophila* sialyltransferase (*DSiaT*). *DSiaT* mutants display cardiac abnormalities including reduced heart rate and arrhythmia, which is consistent with phenotypes previously observed in mammals. We are now using tissue-specific expression and knockdown of *DSiaT* to test if sialylation has an effect on heart physiology through its role in neural control or heart autonomous function. In addition, we are analyzing other genes of the sialylation and protein O-mannosylation pathways, along with their genetic interactions with candidate genes that potentially collaborate with these pathways in regulation of heart development and physiology. We focus on elucidation of pathological mechanisms that underlie heart phenotypes in glycosylation mutants with the main goal to contribute to the better understanding of etiology of cardiovascular diseases. This project was supported in part by the NIH/NS075534 and NS099409 grants to VP.

### (110) Clinicopathological implications to micropapillary bladder urothelial carcinoma of the presence of sialyl Lewis X-decorated mucin 1 in stroma-facing membranes

Tomochika Shinagawa[1,2], Hitomi Hoshino[1], Minekatsu Taga[1,2], Yasuhiro Sakai[1], Yoshiaki Imamura[3], Osamu Yokoyama[2] and Motohiro Kobayashi[1]

*[1]Department of Tumor Pathology, Faculty of Medical Sciences, University of Fukui, Eiheiji, Japan; [2]Department of Urology, Faculty of Medical Sciences, University of Fukui, Eiheiji, Japan; [3]Division of Surgical Pathology, University of Fukui Hospital, Eiheiji, Japan*

Bladder urothelial carcinoma (UC) comprises more than 90% of all bladder cancers. Among several UC variants, micropapillary UC (MPUC) is a rare one with high potential for lymphovascular invasion and subsequent lymph node metastasis. Histologically, MPUC is characterized by the presence of small papillary carcinoma cell clusters surrounded by lacunar spaces. Immunohistochemically, the outer circumference of these clusters, that is, the stroma-facing membrane of carcinoma cells, is reportedly almost invariably positive for mucin 1 (MUC1) protein and to a lesser extent for sialyl Lewis X (sLeX) carbohydrates; however, the clinicopathological implications of theseexpression patterns have not been fully investigated. We performed immunohistochemical analysis of MPUC ($n = 11$) and conventional UC ($n = 57$) for MUC1 and sLeX to determine whether these factors immunolocalized. Dual immunofluorescence staining was also carried out to assess MUC1 and sLeX colocalization. We also performed western blot analysis of Chinese hamster ovary (CHO) cells misexpressing both recombinant epitope-tagged MUC1 and glycosyltransferases enabling sLeX biosynthesis. MPUC samples preferentially exhibited both MUC1 protein and sLeX carbohydrate expression on the stroma-facing membrane of carcinoma cells. Based on univariate analysis, MUC1 expression in that pattern was positively correlated with tumor extension, lymphovascular invasion, lymph node metastasis, disease stage and relatively poor patient prognosis. A comparable sLeX expression pattern also correlated positively with tumor extension and nodal metastasis. Based on multivariate analysis, localization of MUC1 and sLeX on the stroma-facing side of the membrane was positively correlated with lymph node metastasis. Overall, our immunofluorescence findings as well as immunoprecipitation analyses of CHO cell transfectants strongly suggest that MUC1 is a potential scaffold protein for sLeX carbohydrates in MPUC. Both MUC1 and sLeX may cooperatively contribute to MPUC histogenesis and clinicopathological characteristics.

### (111) N-Glycanase 1 Deficiency Triggers Innate Immune Activation Through Dysregulated Mitophagy

Kun Yang and Nan Yan

*Department of Immunology, UT Southwestern Medical Center, Dallas, TX*

The cytosolic N-glycanase 1 (Ngly1) is an evolutionarily conserved de-glycosylating enzyme, which plays a critical role in the quality control of newly synthesized glycoproteins. Misfolded N-glycoproteins retro-translocated from endoplasmic reticulum lumen to cytosol are deglycosylated by Ngly1 prior to proteasome degradation. Mutations in the human *NGLY1* gene are associated with a severe rare congenital disorder characterized by global development delay, neurological abnormalities and liver diseases. The pathogenesis of the *NGLY1*-associated disease remains largely unknown. In this study, we observed an aberrant innate immune activation inboth human and mouse *Ngly1*$^{-/-}$ cells, as evidenced by elevated expression of interferon-stimulated genes that are collectively known as the 'type I interferon gene signature'. Using pharmacological inhibitors and RNAi knockdown, we identified the cytosolic DNA sensing pathway as the key innate immune pathway activated in *Ngly1*$^{-/-}$ cells. We also found that *Ngly1*$^{-/-}$ cells exhibited fragmented mitochondria and mtDNA leakage into the cytosol, which likely serve as the source for self DNA sensed by the innate immunity. Mitochondria are dynamic organelles, and aged or damaged mitochondria are cleared through mitophagy. We found that mitophagy was impaired in the absence of NGLY1. Together, our data reveal the first evidence of immune dysregulation associated with *NGLY1* deficiency, as well as defects in mitophagy that likely lead to accumulation of damaged mitochondria, mtDNA leakage into the cytosol and activation of cytosolic DNA sensing pathway.

### (112) Semi-Automated Identification and MS1-level Quantification of Permethylated Glycan Isomers Separated by RP-HPLC/MS

M. Osman Sheikh[1], Simone Kurz[1], Brent Weatherly[1], Christopher M. West[1,2], Michael Tiemeyer[1,2] and Lance Wells[1,2]

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

High performance liquid chromatography has been employed to enhance the sensitivity of detection and quantification of complex analytes within biological mixtures. For decades, HPLC has been used for the characterization of underivatized and fluorescently-tagged glycans. Due to the rapidly evolving knowledge of the biological significance of glycans, high-throughput methods to identify and quantify released glycans are essential for the advancement of glycomics research, especially in disease-related areas where large numbers of biological samples must be analyzed. Within the past decade, our group and others have attempted to improve on the current methods of separating permethylated glycans coupled to MS-based detection. Here, we report our optimized workflow for LC-MS analysis of permethylated glycans using the Thermo Scientific Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer, including sample preparation, mobile phase optimization, and post-acquisition data analysis. We compared the glycan profiles of biological samples detected by direct infusion MS to those detected by LC-MS and demonstrate baseline separation and quantification of isomeric structures with $MS^n$ fragmentation to aid in confident identification. Using an in-house software solution, in conjunction with *GRITS* Toolbox, we demonstrate semi-automated glycan identification, comparative glycomics analyses, and improved MS1-level quantification by filtering out MS1 scan events that do not correlate with expected glycan isotopic profiles during particular retention times. Thus, our improved workflow vastly increases the accessibility of glycomics without the requirement of a mass spectrometer with high-resolution/accurate mass capabilities. The application of our workflow for the detection and characterization of glycan profiles from biological samples that present unique challenges will be presented. This work was supported in part by grants from the NIH (R21AI123161 and P41GM103490).

### (113) GPQuest 3: A Tool for Large-scale and Comprehensive Glycosylation Analysis on MS data

Yingwei Hu, Weiming Yang, Punit Shah, Shisheng Sun, Minghui Ao and Hui Zhang

*Department of Pathology, Johns Hopkins University*

Glycosylation is one of the most abundant co- and post-translational modifications in eukaryotic proteins, playing critical roles in biosynthesis, structure maintainingand deliveryof proteins. However, glycosylation is also one of the least understood PTMs, and exceptfew heavily studied glycoproteins.The glycosylation site-specific glycans and quantitation of most glycoproteinswith specific glycosylation at each glycositeremain unsolved.Althoughsoftware tools were developed for the data analysis of mass spectra of glycans and intact glycopeptides, there are many analytical challenges still posed by protein glycosylationsuch as quantitative analysis of glycans, and glycosite-specific glycans. As the result, glycosylation of proteins is often missed from the proteomic data. Here, we present an index-based open modification search strategy in GPQuest 3to simultaneously search intact N-linked and O-linked glycopeptides of data generated by the Clinical Proteomic Tumor Analysis Consortium (CPTAC)without the enrichment of glycopeptides in the search space of the complete refseq human protein database and more than 300 glycans. In the preliminary result, we identified thousands ofintact glycopeptides and glycoproteins from the CPTAC breast cancer data that were published and made available through CPTAC data portal.All the identifications were filtered with FDR = 0.01 and stored as spectral librariesfor further review and cross validated with SPEGmethod.The new approach in GPQuest 3is useful to discover hidden and novel glycosylated peptides with glycans attached to specific glycosylation sitesto investigate the glycoprotein expression in tumor cells.

### (114) Role of Protein O-mannosylation in sensory feedback mechanism in Drosophila

Ishita Chandel, Ryan Baker, Naosuke Nakamura, Dmitry Lyalin and Vladislav Panin

*Texas A&M University, College Station, TX, USA*

Protein O-mannosylation (POM) is a posttranslational modification associated with several congenital neuromuscular disorders. POM is mediated in mammals by enzymes called Protein O-Mannosyl Transferases (POMTs), POMT1 and POMT2, which are conserved in metazoans. In *Drosophila*, defects in these enzymes cause a clockwise rotation of abdominal segments in adult flies. Despite several studies of POM functions, the causative mechanisms responsible for these defects are still unclear. To elucidate these mechanisms, we studied the function of *POMT* genes in *Drosophila* embryo. We found that the late *Drosophila* embryo exhibits an abnormal pattern of muscle contractions and abnormal body torsion. We theorized that these phenotypes are possibly linked, and that they might unravel the molecular and cellular mechanisms common for differentstages of development. To understand the molecular basis of the torsion phenotype we analyzed first instar larvae of Dystroglycan (a known O-mannosylated protein) mutant and found that they did not show a body torsion phenotype. This suggested that Dystroglycan is not the only functionally important target of POMT activity. To test the cellular requirements of POMTs, we used a UAS-GAL4 based rescue strategy. We found that the embryo torsion was significantly rescued by expressing POMT enzymes in neurons, including sensory neurons. Thus, we hypothesized that POMTs are likely required in the

PNS to maintain sensory feedback, thus regulating muscle contractions and body posture. To test this hypothesis, we analyzed mutants depleted in sensory neurons and found that they exhibit a torsion phenotype similar to that of *POMT* mutants. We further employed the FLP-OUT strategy and found that *POMT* mutants showed defects of sensory axon projections. Taken together our results suggest that POMTs are required for proper sensory axon targeting, to maintain communication between PNS and CNS neurons (sensory feedback), which affects the pattern of peristaltic contractions and hence body posture. This project was supported in part by NIH/NS075534, NS099409 and CONACYT 2012-037 (S) grants to VP.

### (115) N-Acetylmannosamine (ManNAc) for the Treatment of GNE Myopathy: 18-Month Preliminary Results from a Phase 2 Open-Label Study

Marjan Huizing[1], Bradley Class[2], Melanie Quintana[3], Christina Slota[2,4], Petcharat Leoyklang[1], Ashleigh Glowacki[1], Kennan Bradley[1], Carla Ciccone[1], May Christine V. Malicdan[1,5], Scott M. Berry[3], William A. Gahl[1,5] and Nuria Carrillo[1,2]

*[1]Medical Genetics Branch, NHGRI, NIH, Bethesda, MD 20892; [2]Therapeutics for Rare and Neglected Diseases, NCATS, NIH, Bethesda, MD 20892; [3]Berry Consultants LLC, Austin, TX 78746; [4]RTI Health Solutions, Research Triangle Park, NC 27709; [5]NIH Undiagnosed Diseases Program, Common Fund, Office of the Director, NIH, Bethesda, MD 20892*

GNE myopathy is a rare, autosomal recessive, adult-onset myopathy, caused by deficiency of the rate-limiting enzyme (GNE) of sialic acid (Neu5Ac) biosynthesis. In GNE myopathy, progressive skeletal muscle atrophy decreases strength, function and quality of life in patients. There is no approved therapy. *N*-acetyl-D-mannosamine (ManNAc), an uncharged monosaccharide and the first committed precursor in the sialic acid biosynthetic pathway, is an oral therapeutic candidate that prevents muscle weakness in mouse models of GNE myopathy. Our Phase 1 trial showed that ManNAc is safe and increases sialic acid production in GNE myopathy patients.

We present an interim analysis after 18 months of oral administration of ManNAc at doses of 6 grams twice daily in an ongoing open-label phase 2 study (NCT02346461) of ManNAc in GNE myopathy patients.

Twelve patients were enrolled in two cohorts. Safety, pharmacokinetics, pharmacodynamics, as well as measures of strength, function and patient-reported outcomes were evaluated after administration of ManNAc for 18 months.

Long-term administration of ManNAc appeared safe. Twice daily ManNAc dosing results in a sustained increase in levels of plasma free sialic acid, while ManNAc does not accumulate in plasma. Comparison of muscle biopsies taken at baseline and at 3 months showed significant improved sialylation of membrane glycans after administration of ManNAc. Muscle strength studies at 18-months showing a ~45% slowing in the rate of decline (compared to natural history decline), with a 96% probability that ManNAc slowed the rate of progression of disease. When subjects were asked about their ability to perform tasks or functions, the majority reported their functional ability to be the same or improved.

These findings support further development of ManNAc as a therapy for GNE myopathy.

### (116) Quantitation of Sialylation Status by Lectin Immunofluorescence in Muscle Biopsies

Marjan Huizing[1], Petcharat Leoyklang[1], Bradley Class[2], Colleen Jodarski[1], Carla Ciccone[1], Ashleigh Glowacki[1], William A. Gahl[1,3], Nuria Carrillo[1,2] and May Christine V. Malicdan[1,3]

*[1]Medical Genetics Branch, NHGRI, NIH, Bethesda, MD 20892; [2]Therapeutics for Rare and Neglected Diseases, NCATS, NIH, Bethesda, MD 20892; [3]NIH Undiagnosed Diseases Program, Common Fund, Office of the Director, NIH, Bethesda, MD 20892*

GNE myopathy is a rare, adult onset, muscle disorder of progressive muscle weakness and atrophy, caused by deficiency of the key enzyme in sialic acid (NeuAc) biosynthesis. Effective biomarkers for diagnosis and response to therapy are lacking for this disorder. *Qualitative* lectin staining of muscle biopsies from GNE myopathy humans and mice demonstrated reduced sarcolemmal sialylation, and mouse sarcolemma re-sialylation upon oral therapy with the sialic acid precursor N-acetylmannosamine (ManNAc).

We developed a sensitive and reproducible method to quantify membrane fluorescence in human muscle biopsies, based on imaging entire muscle biopsy sections (rather than having the investigator choose the image area and score it by eye) in Z-stacks and tile scans (~100 tiles/slide) using matching confocal microscope settings (rather than having the investigator determine the imaging conditions). Tile scans were stitched into one image file, used for calculation of membrane fluorescence by specialized, unbiased software in different outputs (Definiens Inc). First, we used fluorescent labeled SNA lectin (binding terminal Neu5Ac in an α2,6-linkage) and antibodies to the muscle membrane protein CAV-3 (caveolin-3) confirming that GNE myopathy muscle biopsies had a lower % of SNA co-localizing with CAV-3 than controls. Next, we demonstrated with SNA/CAV-3 fluorescence quantification that most muscles biopsied after 90 days of oral ManNAc therapy (twice daily as part of our ongoing Phase 2 trial) had significantly more sarcolemmal sialylation than the same muscles biopsied at baseline (11 biceps and 10 lower extremity).

This novel unbiased fluorescence quantitation method demonstrated efficacy of ManNAc therapy for muscle re-sialylation in GNE myopathy and may also be applicable for fluorescence intensity measurements of sarcolemmal components in other muscle disorders.

### (117) GlycoStore: a resource for the exploration and annotation of LC and CE glycomics data

Matthew P. Campbell[1], Sophie Zhao[2], Ian Walsh[2], Jodie L. Abrahams[1], Louise Royle[3], Nicolle H. Packer[1,4], Terry Nguyen-Khuong[2] and Pauline M. Rudd[2]
*[1]Institute for Glycomics, GriffithUniversity, Gold Coast, Australia; [2]Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), Singapore; [3]LudgerLtd., Culham Science Centre, Oxfordshire, United Kingdom; [4]Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia*

GlycoStore (http://www.glycostore.org) is an open access chromatographic and electrophoretic retention database of *N*-, *O*- and GSL glycans characterised from a range of glycoproteins, glycolipids and biotherapeutics. It is a continuation of the GlycoBase project, which addresses many of the technological limitations of GlycoBase, in particular, improving the bioinformatics architecture, enhancing data annotations and connectivity with other resources.

The database is built on publicly available experimental data sets from GlycoBase originally developed in the Oxford Glycobiology Institute and then the National Institute for Bioprocessing Research and Training (NIBRT). It has now been extended to include recent published, and in-house data collections from the Bioprocessing Technology Institute (BTI) and Ludger Ltd.

The database has four levels. The first level brings together annotated glycomics data sourced from a number of analytical platforms including ultra-high performance liquid chromatography, porous graphitized carbon chromatography with MS detection, and capillary electrophoresis with laser induced fluorescence detection. The second level provides access to a growing, curated database of published literature, with a focus on data that has become available over the past five years, filling an information gap between GlycoBase and GlycoStore. This content includes a number of features such as a detailed comparison of serum glycosylation profiles derived from healthy individuals with a number of different diseases, providing insights into how glycosylation changes are associated with health and disease. The third level is a new search tool that allows users to efficiently filter structure entries based on annotated features (e.g. epitopes and mass), category type, and glycoprotein. The last level is the provision of a dedicated Semantic endpoint that provides developers a platform to query and data mine the available content.

Metadata is critical for connecting, finding and reusing glycomics data. Our aim is to create a metadata specification and a glycomic collections registry to facilitate data discovery. We are developing a web-based interface and visualisation tools enabling users to explore GlycoStore, compare data sets, and filter by structural and analytical facets. It contains the largest collection of curated and in-house LC and CE experimental data on glycan structures and associated research literature. We will continue to adapt its data gathering, processing and user interfaces to support on-going developments in separation-MS-based analytical workflows, especially integration with ion-mobility data collections. It also provides the functionality to access/download supporting data, as well as to visualise regions of interest and connections with data available in other UniCarb resources.

### (118) Biochemical and molecular-genetic analysis of two siblings with congenital disorder of glycosylation caused by a novel mutation in ATP6AP1 gene

Nina Ondruskova, Alzbeta Vondrackova, Marketa Tesarova, Tomas Honzik, Jiri Zeman and Hana Hansikova
*Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, the Czech Republic*

**Background:** A boy(P1) born in term was hospitalized for hyperbilirubinemia and hepatopathy at two months of age. Hemanifested with hepatosplenomegaly, coronal hypospadias and wrinkled skin, and died at the age of three months due to progressive liver failure. The mother later gave birth to a healthy girl and two male twins, one of whom (P2) presented with similar complications to P1 and died as 11-month-old.

**Results and discussion:** CDG (Congenital Disorders of Glycosylation) screening test showed hypoglycosylated serum transferrin and apolipoprotein C-III in both patients, pointing to a combined defect of N- and O-glycosylation. Overall hyposialylation of O-glycoproteins was confirmed in fibroblasts from P1 by immunofluorescence with labeled PNA lectin. The following analysis by immunocytochemistry using brefeldin A treatment revealed delayed retrograde Golgi transport. Whole-exome sequencing in P1 identified a novel hemizygous mutation c. 221 T > C (p.Leu74Pro) in exon 2 of X-chromosome-located *ATP6AP1* gene, absent in the father and present heterozygously in the mother. The same mutation was subsequently found in P2 by Sanger sequencing, confirming the diagnosis of ATP6AP1-CDG in the affected siblings. Compared to the previously described cases (11 patients from 6 families), our patients showed a more severe phenotype and manifested with a novel clinical feature of wrinkled skin. Furthermore, peroxisomal disturbance as demonstrated by aberrant ratio of plasma VLCFA and reduced peroxisomal staining of catalase in fibroblasts was observed in P1.

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### (119) Siglec-8 ligands in human airway secretions

Anabel Gonzalez Gil[1], Hyun Sil-Lee[2], Ryan Porell[1], Steve M. Fernandes[1], Jean Kim[2,3] and Ronald L. Schnaar[1]
*[1]Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD; [2]Department of Medicine: Allergy and Clinical Immunology, Johns Hopkins University School of Medicine, Baltimore, MD; [3]Department of Medicine: Otolaryngology,*

*Head & Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD*

Siglecs – sialic acid binding Ig-like lectins – are regulatory molecules expressed on subsets of immune cells where most inhibit inflammation when engaged by complementary sialoglycan ligands on target tissues. Both major allergic inflammatory cells, eosinophils and mast cells, express Siglec-8 on their cell surfaces. When Siglec-8 binds to sialoglycan ligands on tissues, apoptosis of eosinophils and inhibition of mediator release by mast cells is induced, limiting inflammation. Although Siglec-8 has been shown to bind synthetic 6'-sulfated sialyl N-acetyllactosamine, the endogenous Siglec-8 ligands in human airways are still unknown. We previously identified sialylated keratan sulfate chains on aggrecan as Siglec-8 binding ligands extracted from postmortem human airways. The current study demonstrates similar but not identical sialoglycan ligands in fresh human airway secretions. Prior to surgery, nasal and sinus saline lavage was collected from patients. Aliquots of soluble lavage proteins were resolved by composite agarose-acrylamide gel electrophoresis, blotted to PVDF, and probed with Fc-tagged Siglec-8. Of > 20 patient samples, nearly all had a single major Siglec-8 binding species that migrated between 800 and 1000 kDa, with most also having a minor binding species at ≥4000 kDa. Binding of Siglec-8 to each species was reversed by treatment with sialidase or keratanase. Immunoblotting the same blots with an antibody against highly sulfated keratan sulfate chains (5D4) revealed binding that co-migrated with Siglec-8 binding. We conclude Siglec-8 ligands present in human airway secretions have similar properties as those extracted from human trachea where the sialoglycoprotein ligand is a high molecular weight protein and the glycan ligand is a sialylated and highly sulfated keratan sulfate chain. Knowledge of the structures and functions of Siglec-8 sialoglycan ligands in the human airways may provide enhanced leads for drug development to treat inflammatory airway diseases. Supported by the Lung Inflammatory Disease Program of Excellence in Glycosciences (http://lidpeg.org) under the auspices of the National Heart, Lung and Blood Institute (P01HL107151).

### (120) Low Level Pancreatic Beta Cell Sialylation in the Onset of Autoimmune Diabetes

Douglas M. Heithoff, Damien Restagno, Won Ho Yang, Peter V. Aziz and Jamey D. Marth

*Center for Nanomedicine, Sanford-Burnham-Prebys Medical Discovery Institute, University of California-Santa Barbara, Santa Barbara, California, USA*

The pathogenesis of tissue-specific autoimmune disease reflects innate or acquired defects in immunological tolerance but remains poorly understood. In Type 1 diabetes (T1D), a cell type-specific defect in immunological tolerance results in the destruction of pancreatic beta cells. In the past decade, multiple studies have linked the post-translational modification of proteins by sialyltransferases with mechanisms of immunological tolerance. Sialyltransferases generate sialic acid linkages on most mammalian cell surfaces modulating autoimmunity and immunological tolerance. We have noticed that normal pancreatic beta cells have relatively low levels of sialic acid linkages among cell surface glycans. This intrinsic low level of sialic acids may be advantageous in normal physiological contexts but disadvantageous in the presence of a dysfunctional immune system. To investigate this hypothesis, we chose to initially study the Non-Obese Diabetic ShiLT/J (NOD) mouse because of its well-defined disease signs that include spontaneous insulitis progressing to beta cell destruction, and because of the large body of immunological work that has been achieved using this animal model of autoimmune diabetes. We have generated and analyzed multiple transgenic NOD mice bearing increased expression of sialic acids on pancreatic beta cell proteins. Our findings reveal that augmentation of sialic acid linkages protects from insulitis, hyperglycemia, and the immunological destruction of pancreatic beta cells.

### (121) TREX1 prevents the accumulation of an endogenous bioactive disaccharide associated with autoimmunity

Charles S. Fermaintt[1], Mark A. Lehrman[2] and Nan Yan[1]

*[1]Department of Immunology; [2]Department of Pharmacology,UT Southwestern Medical Center, Dallas, TX*

TREX1/DNase III is an endoplasmic reticulum (ER) tail-anchored exonuclease whose mutations are associated with autoimmune diseases characterized by elevated levels of immune gene transcripts. Most of the disease mutations located near the C-terminal tail-anchor region of TREX1 are frame-shifts (fs) (E.g. V235fs) that result in TREX1 mislocalization, without affecting TREX1 DNase function. Recently, we found that the TREX1 tail-anchored region modulates the oligosaccharyltransferase (OST) preference for LLO hydrolysis to generate free oligosaccharides in a switch like manner, through the interaction with the OST subunits Ribophorin 1 (RPN1) and DDOST. Studies using TREX1-V235fs (disease causing mutation) patient lymphoblasts and a TREX1-V235fs knock-in mouse model also revealed elevated levels of free oligosaccharides and immune disorders. Structural analysis on the TREX1-V235fs free oligosaccharides revealed that they differ from typical N-glycans structures with high accumulation of mannosyl tetra, tri and disaccharides. Through size exclusion fractionation, we determined that the structure responsible for the bioactivity is a disaccharide composed of Mannose (Man) and N-acetylglucosamine (GlcNAc) with a β(1–4) linkage. We also determine that OST's free oligosaccharides are the precursors of the Man β(1–4) GlcNAc disaccharide and that these species are generated through the canonical lysosomal degradation pathway. Together, we propose a molecular mechanism where TREX1 fs mutations cause accumulation of a bioactive disaccharide that triggers immune activation which could lead to immune disorder such as retinal

vasculopathy with cerebral leukodystrophy (RVCL) and systemic lupus erythematosus (SLE).

**(122) Inhibition of Notch signaling using fucose analogs**

Huilin Hao[1], Michael Schneider[2], Hideyuki Takeuchi[1], Peng Wu[3] and Robert S. Haltiwanger[1]

*[1]Complex Carbohydrate Research Center, University of Georgia; [2]Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-5215; [3]Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037*

Notch is a cell surface receptor involved in cell-cell communication that has essential roles in many cell-fate related events like differentiation, proliferation and survival. Defects in Notch signaling can lead to a variety of human disorders including cancer. Accumulating evidence supports the pro-oncogenic function of Notch in a wide range of human malignancies, especially in T-cell acute lymphoblastic leukemias (TALL). Developing inhibitory agents targeting the Notch pathway has been intensively investigated. However, the initial efforts of targeting Notch by γ-secretase inhibitors (GSIs) has failed in clinical trials because of serious side effects. Novel strategies for designing agents for Notch-targeted therapeutics is needed. The Notch extracellular domain (NECD) is constituted with up to 36 tandem epidermal growth factor-like (EGF) repeats that are heavily glycosylated. Proper glycosylation of EGF repeats in the NECD is required for the activation of Notch signaling, especially *O*-fucosylation mediated by Protein *O*-fucosyltransferase 1 (POFUT1). Blocking *O*-fucosylation has been proven to destabilize EGF repeats and thereby affect Notch trafficking. Towards this end, we screened L-fucose analogs to find potential inhibitors for Notch *O*-fucosylation. Two analogs, 6-alkynyl and 6-alkenyl fucose, were shown to be potent Notch inhibitors in a ligand selective manner, inhibiting Delta-mediated, but not Jagged-mediated, Notch activation. Both analogs were utilized as substrates of POFUT1 and incorporated directly into Notch EGF repeats in cells, blocking the binding of Notch1 to Delta ligands. We are now testing these analogs against Notch receptors with activating mutations found in TALL. Our data suggests that fucose analogs represent a novel approach to developing Notch-targeted therapeutics. This work was supported by NIH grant GM061126.

**(123) Endogenous galectin-3 promotes muscle repair**

Lilian Cataldi Rodrigues[1], Daniel Giuliano Cerri[1], Vani Maria Alves[2], Martin K. Amstalden[1], Sean R. Stowell[3], Richard D. Cummings[4] and Marcelo Dias-Baruffi[1]

*[1]Faculty of Pharmaceutical Sciences of Ribeirão Preto - University of São Paulo, Ribeirão Preto, São Paulo, Brazil; [2]Cell and Molecular Biology and Pathogenic Bioagents – Ribeirão Preto Medical School – University of São Paulo, Brazil; [3]Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322, USA; [4]Department of Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02115, USA*

Skeletal muscle has an intrinsic ability to self-repair, and throughout life muscle is constantly repaired after daily micro-lesions. After injury, inflammatory responses are positively associated with muscle repair. In the repair process many proteins are involved, which trigger important signaling pathways that connect muscle homeostasis and immune responses. However, whereas there is tantalizing evidence that carbohydrate-binding proteins, such as galectins, may be involved in muscle repair, there is little known about the role of Gal-3, a ubiquitously expressed multifunctional β-galactoside-binding protein, in repair of skeletal muscle. Here we show that Gal-3 is highly expressed in gastrocnemius from *mdx* mice, a model for Duchenne Muscular Dystrophy, the most common dystrophinopathy, but Gal-3 is not detectable on skeletal muscle from wild-type mice. Interesting, using healthy mice in an acute murine model of muscle injury by injecting $BaCl_2$, Gal-3 expression was detectable, peaking within 3 days post-lesion and returning to undetectable levels following resolution of the muscle lesion. Gal-3 has a pleiotropic distribution in damaged muscle, as we detected it using immunohistochemical approaches in myonuclei, sarcolemma and sarcoplasm within muscular tissue from *mdx* mice, which exhibits a similar distribution to that found in $BaCl_2$ injured-muscle from wild-type mice. To further assess the mechanistic role of Gal-3, we induced acute muscle injury in Gal-3 knock-out mice - Lgals3(-/-). The analysis of gastrocnemius indicated a large extent of muscle damage in Lgals3(-/-) mice 3 days after a single $BaCl_2$ injection and even past 28 days, indicating that endogenous Gal-3 is needed for the normal muscular repair processes. Lgals3(-/-) gastrocnemius differed from Lgals3(+/+) muscle in that the former exhibited: (i) persistence of inflammatory infiltrate even after 28 days; (ii) high amounts of collagen deposition in between the myofibers and (iii) some site with lipogenesis; (iv) higher and continuous expression of osteopontin, a critical protein of the tissue repair. These results indicate that endogenous Gal-3 contributes to muscle repair after injury. Understanding the mechanisms and factors that are responsible for muscle repair will facilitate the development of novel therapeutics for dystrophinopathies. *Key words:* Galectin-3, skeletal muscle repair, *mdx* mice.

**(124) Identification and Functional Characterization of Genomic-Glycosylation Aberrations in Human Cancers**

Carman K.M. Ip[1], Xinxin Peng[2], Patrick K.S. Ng[3], Kang J. Jeong[1], Han Liang[2] and Gordon B. Mills[1,3]

*[1]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [2]Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [3]Sheikh Khalifa Bin Zayed Al Nahyan*

*Institute for Personalized Cancer Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, USA*

Protein function is regulated by post-translation modifications that are thus key contributors of tumor initiation and progression. Accumulating evidence implicates altered glycosylation in tumorigenesis as well as therapeutic responses. Thus, a better understanding of the role of protein glycosylation in tumor pathophysiology may provide new insight for the development and implementation of effective therapeutic strategies. Genomic mutations could potentially create or disrupt glycosylation sites; thus, altering the protein conformation, function, or localization. However, the potential contribution of genomic aberrations in tumors to altered glycosylation sites has not been systematically evaluated. The aim of this study is to identify patient-derived missense mutations that could lead to a loss or gain of N-glycosylation due to disruption or creation of the N-glycosylation consensus motif Asn-X-Ser/Thr/Cys, where X is any amino acid except proline, and to study their functional consequences on tumor behavior.

Missense mutations that disrupt or create N-glycosylation consensus sequences were computationally identified from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) sequence data. Potential aberrations in N-glycosylation sites that locate in the extracellular domain of secreted or membrane bound proteins, where N-glycosylation most frequently occurs, were used as a filter. Extensive missense mutations that potentially disrupt (16,156) or create (24,882) N-glycosylation consensus sequences were identified. 3144 potential loss of N-glycosylation site mutations are annotated in UniProtKB/Swissprot as N-glycosylation sites based on experimentally verification or prediction by 'NetNGlyc' or PROSITE pattern predictors. To functionally characterize the role of aberrations in N-glycosylation sites in tumor initiation, over 40 potential N-glycosylation site altering mutations in receptor tyrosine kinase and cadherin family proteins were created assessed for transformation activity using Ba/F3 and MCF10A cells. Several of the aberrations alter transforming activity as well as protein stability, localization and therapeutic sensitivity validating the approach and the importance of mutations in N-glycosylation sites in tumorigenesis.

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## (125) RECOMBINANT ANTIBODY FRAGMENTS AGAINST SYNTHETIC GLYCOPEPTIDE MIMICKING CANCER MUCINS: A PROMISING TOOL FOR DIAGNOSIS AND DRUG DELIVERY SYSTEMS

Thais Canassa De Leo[1], Marcelo M. Brígido[2], Andrea Q. Maranhão[2], Vanessa L. Campo[1] and Marcelo Dias-Baruffi[1]

*[1]School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Brazil; [2]Institute of Biological Sciences, University of Brazilia, Brazil*

Cancer is a severe disease associated with alterations in the health molecular glycosylation patterns that lead to pathophysiologic consequences and to the discovery of cancer-biomarkers. Hypoglycosylated MUC1 (transmembrane glycoprotein Mucin 1) is an important tumoral marker for several types of cancer. Two of the most important challenges in cancer clinical research are the development of precise diagnostic and therapeutic strategies that allow the targeting of anti-tumor drugs direct to the cancer cell and not to healthy cells. Thus, the development of site-specific drug delivery and diagnostic systems based on tumoral markers are important approaches in the fight against cancer. Here we described DNA sequence, cloning, and expression of selected human recombinant antibody fragments against synthetic glycopeptides based on MUC1. Accordingly, the synthetic glycopeptide NHAcSer-Ala-Pro-Asp-Thr[βGalNAc]-Arg-Pro-Ala-Pro-Gly-BSA, mimetic of hypoglycosylated MUC1, was obtained by solid phase synthesis followed by conjugation reaction with the carrier protein BSA. This conjugated glycopeptide was then immobilized on ELISA microplates for selection of antibodies fragments from a human antibody library, by Phage Display. Coding genes of selected antibodies were sequenced in Illumina Miseq system and analyzed using a novel platform (ATTILA). The results indicated that Phage Display selection for anti-synthetic glycopeptides was very efficient, since the last 4$^{th}$ round was enriched around 10,000-fold relative to initial round. Also, the most enriched sequences were associated with the higher affinity to tumor-associated antigen. The best two sequences, containing canonic human antibody sequences, were cloned into a pET29a expression vector and used to transform competent *E. coli* cells. We further propose to use the soluble anti-MUC1 glycopeptide antibody fragments to develop novel chemotherapy delivery and cancer cell detection systems.

Keywords: MUC1, Glycopeptide, Phage Display, Antibody, Cancer

## (126) Endogenous galectin-3 impairs neutrophil migration and increases susceptibility in a murine model of severe polymicrobial sepsis

Raphael G. Ferreira[1], Lilian C. Rodrigues[2], Daniele C.B. Nascimento[1], Alexandre Kanashiro[1], Paulo H. de Melo[1], Vanessa F. Borges[1], Aline Gozzi[3], Marcos C. Borges[3], Sean R. Stowell[4], Richard D. Cummings[5], Marcelo Dias-Baruffi[2], Fernando Q. Cunha[1] and José Carlos Alves-Filho[1]

*[1]Pharmacology Department, Ribeirão Preto Medical School, University of São Paulo-Brazil.; [2]Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo-Brazil.; [3]Department of Internal Medicine,Ribeirão Preto Medical School, University of São Paulo-Brazil.; [4]Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322, United States of America.; [5]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, United States of America*

Sepsis is an overwhelming systemic inflammation resulting from an uncontrolled response to infection that causes extensive tissue damage, organs dysfunction and eventually death. In addition, defective neutrophil recruitment to the microbial focus is associated with poor outcome in sepsis. Recent studies indicate that host endogenous molecules released in response to tissue damage are involved in the pathophysiology of sepsis. Here we show that galectin-3 (Gal-3), an endogenous carbohydrate-binding protein that displays several immune modulatory activities, plays a critical role in sepsis progression. Serum Gal-3 concentration is increased in a murine model undergoing lethal experimental sepsis induced by cecal ligation and puncture (CLP). This correlates with our detection of higher serum levels of Gal-3 in sera from patients with septic shock, as previously reported by others. Interestingly, Gal-3 knockout mice (Lgals3 (-/-) mice) are more resistant to severe sepsis induced by CLP, presenting lower levels of biochemical markers for organ injury and dysfunction than those observed in wild type mice (Lgals3( + / + ) mice). Moreover, Lgals3(-/-) mice show increased number of neutrophils in the primary focus of infection and reduced bacterial loads in the peritoneal cavity, blood and lungs. Mechanistically, blood neutrophils from septic mice show higher levels of surface-bound Gal-3 than neutrophils from control mice. The deficiency of Gal-3 in Lgals3(-/-) mice is associated with increased rolling and adhesion of these cells in mesenteric venules. Our results indicate that endogenous Gal-3, released during sepsis, hinders neutrophil recruitment into the infectious focus, which promotes bacterial spread and negative sepsis prognosis. *Key words:* sepsis, Galectin-3, neutrophil.

### (127) Mass spectrometry analysis of adeno-associated virus glycan receptor expression in nigrostriatal pathway in aging rats

Rekha Raghunathan[1], Nicole Polinski[3], John D. Hogan[4], Joshua Klein[4], Kshitij Khatri[2], Chun Shao[2], Caryl Sortwell[3] and Joseph Zaia[2]

*[1]Department of Molecular and Translational Medicine, Boston University; [2]Department of Biochemistry, Boston University; [3]Department of Translational Science and Molecular Medicine, Michigan State University; [4]Department of Bioinformatics, Boston University*

*Introduction:* Parkinson's disease is a neurological disorder characterized by the lack of functional dopaminergic neurons in the nigrostriatal pathway in the brain. Current treatments provide only symptomatic relief. Gene therapy has the potential to improve the dopamine production in Parkinson's patients. Adeno-associated viruses are vectors of choice in gene therapy due to their well characterized safety and efficacy profiles. Significantly, all primary receptors of the virus are glycans. While gene therapy has been successful in preclinical models of the disease, clinical trials in humans have failed.

*We hypothesize that age related changes in glycan receptors of heparan sulfate (HS) proteoglycan (receptor for rAAV2) results in poor adeno-associated virus binding in the nigro-striatal region of the brain, affecting transduction and gene delivery.*

To test our hypothesis we focus on analyzing the striatum and substantia nigra for changes in heparan sulfate and proteomic signatures in aging rat model.

*Method:* We analyzed glycan classes and proteins from rat brain histological sections using liquid chromatography-mass spectrometry in the nigrostriatal pathway.

*Results:* We observed different HS disaccharide profiles in aged (20 months) compared to young (3 month) rat for brain region specific profiles in striatum versus substantia nigra. In particular, the receptor for the virus is NS, 6 S sulfated patterns with corresponding disaccharide compositions of D0S6 and D2S6. We observed that the abundance of this receptor is lower in old compared to young brains. We also observe that overall HS levels is lower in aged striatum compared to young brains. Further, proteomic profiling of these brain regions led to the observation of proteins differentially expressed in aging nigrostriatal pathway, quantified by label-free proteomics. In particular, the co-receptor for the virus is CD9, is differentially expressed in aged striatum. This is consistent with the reduction in transduction levels of rAAV2/2 in aged brains. Further studies in human brain will enable mass spectrometry guided design of viral vectors for success in gene therapy in clinical trials.

### (128) A Well-Characterized Human Chimeric Anti-Tn Monoclonal Antibody with Cytotoxic Potential

Yasuyuki Matsumoto[1], Matthew R. Kudelka[2], Melinda S. Hanes[1], Sylvain Lehoux[1], Jamie Heimburg-Molinaro[1], David F. Smith[2], Tongzhong Ju[2] and Richard D. Cummings[1]

*[1]Department of Surgery, Beth Israel Deaconess Medical Center - Harvard Medical School; [2]Department of Biochemistry, Emory University School of Medicine*

The Tn antigen is an abnormal mucin type O-glycan (GalNAc-alpha1-O-Ser/Thr) expressed in a majority of carcinomas. Expression correlates with metastasis and poor survival, and is suspected to influence tumorigenesis and metastatic transformation. To better define the expression and function of the Tn antigen, as well as develop new diagnostic reagents, we prepared a recombinant anti-Tn IgM and IgG from a murine monoclonal. The recombinant anti-Tn antibody generated a murine IgM framework is termed ReBaGs6 and the recombinant human chimeric anti-Tn antibody generated using a human IgG1 framework is termed Remab6. Neither of these antibodies cross-react with Tn glycans terminating in GalNAc, such as blood group A and Forssman antigen. Remab6 was characterized with Tn glycopeptide and glycan microarrays and a myriad of biochemical and biological techniques. Results from Tn glycopeptide and CFG glycan microarray studies demonstrated that Remab6 recognizes di- or tri-Tn clustered structures on mucin tandem repeats, and shows little interaction with similar glycan structures expressing terminal GalNAc. Using glycosyltransferases to remodel a

glycopeptide array, we found that Remab6 is highly specific for Tn over the related and elongated products named T and STn antigens. Flow cytometry, western blotting, and immunofluorescence results showed that Remab6 recognizes human colon, breast, and gastric cancer cell lines and leukemic cell expressing the Tn antigen, but does not bind to their Tn-negative counterparts. Antibodies that are highly specific for tumor cells have clinical uses as immunotherapies. Towards this goal, we found that Remab6 exhibits antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity *in vitro*. Immunohistochemistry clearly distinguished staining in Tn+ tissues in IEC-*Cosmc* knockout mice and human cancer cell block sections. These data indicate that Remab6 is specific for the Tn antigen and is useful for biochemical characterization of cancer cell lines and tumor sections, and may be a promising novel therapeutic agent for targeted cancer treatment. [This work was supported by NIH Grant CA168930 to RDC and TJ and P41GM103694 to RDC.]

## (129) GRITS Toolbox 1.2 - New features in our freely available software system for processing and archiving of glycomics mass spectrometry data

Rene Ranzinger, Brent Weatherly, Sena Arpinar, Mindy Porterfield, Lovina Dmello, Michael Tiemeyer and William S. York

*Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, USA*

In recent years the technical advances in mass spectrometry (MS) have led to a steady improvement in accuracy and throughput of MS-based glycomics analysis. In addition, increased recognition of the importance of glycans in biological processes and disease has created growing interest in the study of these molecules and their interaction with other molecules.

These trends have motivated us to implement GRITS Toolbox as an extendable software system for processing, interpreting and archiving of glycomics MS data. This platform allows glycomics MS data (MS/MS, LC-MS/MS, TIM or MS profile data) to be loaded together with metadata describing the project, the analyzed sample and experimental procedures utilized. The integrated data interpretation module, called Glycomics Elucidation and Annotation Tool (GELATO), annotates experimental data with glycan and glycan fragment structures from candidate databases. An extensive set of graphical user interfaces can then be used to visualize, review, modify and export the annotated data or to compare the annotations of different samples side by side.

In the recent release of GRITS Toolbox 1.2 multiple new features have been added to improve usefulness and user friendliness of the application. (1) The preinstalled manually curated default glycan databases (mammalian N-Glycans, O-Glycans, and GlycoSpingolipids) can be extended with additional structures or replaced with user defined databases. (2) Key fragment peaks can be added to the annotation overview table to help in the selection of the right candidate structures. (3) User defined preferences can be used to customize and store default data analyses settings.

In addition several research groups have started to develop own extensions (plugins) of GRITS Toolbox to process other types of data (MS data of glycosphingolipids, MS data of per-methylated glycopeptides, and glycan array data) of which several will be presented at the annual meeting of the Society of Glycobiology.

The current version of the software system is freely available from our project website: http://www.grits-toolbox.org.

## (130) DIFFERENTIAL SIALYLATION AND POLYSIALYLATION IN SKIN AND ORAL MUCOSAL WOUND HEALING

Veronica A. Haywood[1], Lin Chen[1], Karen J. Colley[2] and Luisa A. DiPietro[1]

[1]*Center for Wound Healing & Tissue Regeneration, College of Dentistry, University of Illinois, Chicago, IL;*
[2]*Department of Biochemistry, College of Medicine, University of Illinois, Chicago, IL*

As compared to the skin, oral mucosal wound healing is characterized by a well-orchestrated immune and angiogenic response along with less scar tissue formation. Investigating differences between these tissue sites can suggest pathways and molecules that are critical to scar formation. While polysialylation and sialylation are pivotal in both regeneration and fibrosis, no study has examined their presence or role during skin and oral mucosal wound healing. The objective of this study is to determine whether α2,3-, α2,6-, and α2,8 poly-sialylation is differentially regulated in skin and tongue wounds during wound healing.

Affymetrix microarrays of excisional skin and tongue wounds harvested from Balb/c mice ($n = 3$) at baseline, 6 and 12 hours, and days 1, 3, 5, 7 and 10 post-wounding were probed for genes related to sialylation and polysialylation. Among the genes examined, those encoding α2,3 sialyltransferases were primarily downregulated 12 h post-wounding in the tongue and skin and remained downregulated throughout skin wound healing (FDR < 0.05, FC≥1.5). Genes responsible for GalNAc α2,6 sialyltransferase activity had peak upregulation at 12 h in the tongue and day 1 post-wounding in the skin (FDR < 0.05, FC≥2) and remained upregulated through day 5 in the skin. Additionally, polysialyltransferases, ST8Sia-II and ST8Sia-IV, demonstrated peak upregulation on days 7 and 1, respectively, in the skin (FDR < 0.05, FC≥2) and days 3 and 5, respectively, in the tongue (FDR < 0.05, FC≥1.5).

Lectin blot analysis and endo N trap isolated proteins from the skin and tongue at baseline and days 3 and 10 post-wounding were in agreement with the trends of the microarray data. Skin and tongue wounds displayed decreased α2,3 sialylation and increased polysialylation 3 days post-wounding ($p < 0.05$). Additionally, the skin and tongue displayed differential

expression of α2,6 sialylated proteins during the inflammatory phase of healing (day 3) and differentially expressed α2,3 sialylated proteins during the remodeling phase (day 10) ($p < 0.05$).

Immunohistochemical analysis confirmed that α2,3 linked sialic acids were reduced in the extracellular matrix of skin wounds 3 days post wounding. Furthermore, 12F8 staining revealed increased polysialic acid expression localized to the regenerating epithelium in both skin and tongue wounds 3 days post-wounding. The tongue additionally demonstrated staining of cells located within the papillary neurovascular complex.

These findings reveal that skin and tongue wounds differentially express sialylation related genes which coincides with downstream changes in sialylation and polysialylation. Furthermore, enhanced downregulation of α2,3 sialylation in skin wound healing, the differential expression of polysialyltransferases, and the localization of polysialic acids could play a role in the differential formation of scar tissue observed between skin and oral mucosal wounds. Further research is required to establish causation.

### (131) Beyond ERAD: N-glycanase will bring you to tears

Mitali A. Tambe, Bobby G. Ng and Hudson H. Freeze
*Sanford Burnham Prebys Medical Discovery Institute*

Patients with mutations in *NGLY1* cannot make tears (alacrima), have global developmental delay, movement disorder and liver dysfunction. N-glycanase 1 (NGLY1) deglycosylates misfolded N-glycosylated proteins in the cytoplasm as part of the ERAD pathway prior to their proteasomal degradation. Surprisingly, NGLY1-deficient patient cells do not accumulate cytoplasmic misfolded N-glycoproteins, suggesting a more complex function. Interestingly, new studies show NGLY1 is needed for transcription factor NFE2L1/NRF1 activation and normal mitochondrial function.

Using WT and NGLY1-deficient mouse embryonic fibroblasts (MEFs) we found that NGLY1-deficient cells were resistant to hypotonic lysis compared to WT. The same was seen in NGLY1-patient fibroblasts. Additionally, MEFs deficient in both NGLY1 and ENGase (another deglycosylation enzyme) were even more resistant to hypotonic lysis compared to NGLY1-deficient cells. Water influx and cell swelling precedes cell lysis. We found that NGLY1-deficient MEFs swell slower than WT MEFs. Since aquaporins (AQP) transport water, we hypothesized that AQP levels might be disrupted in NGLY1-deficient cells. We found both AQP1 mRNA and protein were reduced in NGLY1-deficient MEFs. shRNA knockdown of AQP1 in WT MEFs decreased hypotonic lysis, suggesting AQP1 is associated with hypotonic lysis. NGLY1 shRNA and CRISPR studies confirmed that NGLY1 regulates AQP1 levels and hypotonic cell lysis. Preliminary studies show that complementing NGLY1-deficient MEFs with NGLY1 increases AQP1. Current efforts are directed towards confirming this. 13 types of AQP are expressed in different cell types. Our preliminary data suggest that AQP11 levels are disrupted in NGLY1-deficient patient cells and CRISPR cells, suggesting that NGLY1 might regulate different types of AQP.

We have identified a novel function of NGLY1, i.e. to directly/indirectly regulate AQPs. This finding may relate to NGLY1-deficient patients' inability to make tears. Future efforts will try to identify NGLY1-dependent transcription factors responsible for regulating AQPs. This work is supported by the Bertrand Might Research Fund and NGLY1.org.

### (132) GPTwiki - A Glycopeptide Transition Database for Quantitative Glycoproteomics using SWATH

Nathan J. Edwards, Miloslav Sanda and Radoslav Goldman
*Georgetown University*

Quantitative glycoproteomics for the discovery of glycoprotein cancer biomarkers has long been stymied by laborious quantitation techniques which measure the abundance of glycans and proteins independently, that require a glycan or glycopeptide hypothesis, or which lack analytical precision and specificity. Recent work in the Goldman lab has demonstrated the efficacy of a high-throughput, hypothesis generating mass-spectrometry workflow for quantitative glycoproteomics called Glycopeptide SWATH, using glycopeptide transitions generated by soft CID fragmentation.

To facilitate the use of this workflow by analytical core-facilities and to ease adoption in the glycobiology community, we are developing a community resource, GPTwiki, of glycopeptide transitions suitable for use in glycopeptide data-independent acquisition, aka SWATH, quantitation workflows applied to complex mixtures of glycoproteins. Glycopeptide transitions can be entered manually, extracted from data-dependent or targeted CID/ETD tandem mass-spectra of glycopeptides, or mined from SWATH analyses of enriched glycoprotein digests. GPTwiki glycopeptide transitions can be browsed manually, facilitating targeted analyses using MRM quantitation workflows, or downloaded in TraML or TSV format for use by SWATH analysis software. Finally, GPTwiki provides a cloud-based Galaxy workflow implementation of OpenSWATH using GPTwiki transitions for the analysis of large-scale glycopeptide SWATH datasets.

### (133) Exploring the role of Nrf1 in NGly1 deficiency

Ulla I.M. Gerling-Driessen[1], Frederick M. Tomlin[1], C.J. Cambier[1], Yi-Chang Liu[1] and Carolyn R. Bertozzi[1,2]
*[1]Department of Chemistry, Stanford University, Stanford, California 94305, United States; [2]Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, United States*

Recently a rare inherited congenital disorder, N-Glycanase 1 (NGly1) deficiency, caused by heterozygous inactivating mutations in the *ngly1* gene has been discovered. Patients suffering from NGly1 deficiency exhibit a spectrum of symptoms, such as global developmental delay, hypotonia, seizures, peripheral neuropathy, alacrima and liver abnormalities. NGly1 is thought to function as a key component of the ER-associated degradation (ERAD) machinery.

By catalyzing the de-N-glycosylation of glycoproteins it plays an important role in degradation of all misfolded glycoproteins that are retro-translocated from the ER to the cytosol for proteasomal degradation. With this proposed central role in protein turnover it is difficult to comprehend how NGly1 deficient patients even survive.

NGly1 deficient cells have been found to lack a sufficient proteasome function. Therefore, we wondered if the physiologically relevant role of NGly1 during human development might be more specialized than previously thought.

Our hypothesis is that a single NGLy1 substrate might be dependent on de-N-glycosylation for its function and could therefore be the underlying mechanism mediating disease pathologies. The transcription factor Nuclear Factor Erythroid-2 Related Factor 1 (NFE2L1, also called Nrf1), a member of the "cap'n'collar" (CNC) bZIP family is the main regulator of proteasomal subunit gene expression. It is involved in many vital metabolic pathways, including the transactivation of antioxidant enzymes and phase 2 detoxification and thus regulates the proteostasis of cells. Its activation occurs via the ERAD-pathway and involves retro-translocation from the ER to the cytosol, de-N-glycosylation, and partial proteolytic processing to generate the active forms of Nrf1, which migrates to the nucleus to effect gene transcription. Under basal conditions, Nrf1 is continually targeted for proteasomal degradation. However, under conditions where proteasome activity is compromised, such as during treatment with proteasome inhibitors, deglycosylated Nrf1 accumulates and exerts its nuclear functions, including transactivation of proteasomal subunit genes (bounce back).

Importantly, the phenotypes of tissue-specific knockdowns (CNS, liver, bone) overlap with some NGly1-null pathologies. We found that the correct processing, subcellular localization and activity of Nrf1 requires functional NGly1. On this basis, we hypothesize that impaired de-N-glycosylation of Nrf1 in the absence of NGLy1 results in an abrogated bounce back response that in turn contributes to the disease symptoms associated with NGLy1 deficiency and that a rescue of functional Nrf1 might be a valuable therapeutic avenue to treat the symptoms of NGly1 deficiency.

### (134) Glycosylation impacts antibody Fc receptor function and is tuned by the immune system

Nickita Mehta[1], Kevin B. Chandler[2], Catherin E. Costello[2] and Galit Alter[1]

*[1]Ragon Institute of MGH, MIT and Harvard, Cambridge, MA 02139; [2]Center for Biomedical Mass Spectrometry, Boston University School of Medicine, Boston, MA*

Antibodies and their receptors are critical glycoproteins that bridge the innate and adaptive immune systems to provide protection during infection. The isotype andsubclass of the antibody, and post-translational modifications like glycosylation on the CH2 domain, are the two known modifications of the Fc domain that program antibody effector function. Allisotypes/subclasses of antibodies have distinct functions, respond to a wide range of infections and are expressed in different tissues. It has been established that addition and removal of certain glycan structures can influence the potency of antibodies, highlighting the importance of understanding antibody glycosylation. Although IgGs have a single *N*-glycosylation site and are very well studied, other antibody isotypes like IgA and IgM, that are the first responders in certain diseases, have multiple sites ranging from 2–5 sites/monomer of antibody, and very little is known about their glycosylation. Here we employ an LC-MS method to isolate, enhance and separate very small amounts of purified complex glycoprotein to identify the glycan reportoire and site occupancy of bulk serum antibodies. High throughput analysis using our in-house automated glycopeptide analysis software helped elucidate the diversity of site-specific *N*-linked glycan signatures from digestion of purified isotypes, ranging from high mannose, complex and triantennary glycans on the different antibody isotypes. In addition, we were also able to determine the various glycoforms on a single site within the same serum sample as well as assess their site occupancy. Using this method, we have established the N-linked glycan repotoire for bulk IgG1-4, IgA1 and IgA2 and IgM in healthy patients, including low abundant antigen-specific antibodies. Understanding antigen-specific glycan profiles gives us a platform to study the impact of glycosylation mediated effector functions. This ultimately aids in defining disease-specific glycan signatures for different isotypes to help tune antibodes to induce protection.

### (135) DANGO: An MS Data Annotation System for Glycolipidomics

Masaaki Matsubara, Mayumi Ishihara, Michael Tiemeyer, Kazuhiro Aoki and René Ranzinger

*Complex Carbohydrate Research Center, University of Georgia*

Glycosphingolipids (GSLs) are comprised of glycans attached to a ceramide lipid moiety and have been shown to contribute to intra- and intercellular events that influence multiple biological processes. GSL expression is spatially and temporally regulated during development and dysregulated during the progression of many diseases. Altered GSL biosynthesis or degradation results in severe human disorders. Most current glycomics approaches for the characterization of GSL structure utilize enzymatic digestion to release the hydrophilic glycan from the hydrophobic lipid. While this approach simplifies the complexity of the analyte, it blinds the analyst from detecting lipid heterogeneity and, thereby, ignores the biological importance of the lipid portion of the GSLs. Thus, we are developing, a sensitive, robust and comprehensive methodology for the analysis of intact GSL structures using mass spectrometry. Development of highly sensitive, rapid, and facile methods for analyzing the entire GSL structure harvested from biological samples could catalyze broader participation by non-glycoscientists in further understanding GSL structures and functions.

This newly developed methodology is complemented by a semi-automated annotation software tool that employs curated databases for structural assignments. The software supports high throughput MS data analysis by significantly reducing the time required for annotation and interpretation. We are developing our GSL annotation software, DANGO (Data ANnotation system for GlycolipidOmics), as an extension (plugin) to the GRITS-Toolbox (http://www.grits-toolbox.org). GRITS-Toolbox is freely available software for processing and archiving glycomics data. The Toolbox includes an integrated annotation module, GELATO, for annotating glycomics data. Implementing the DANGO as a plugin to GRITS Toolbox allows us to reuse the GELATO functionality for the glycan portion of the GSL and also allows us to investigate and visualize ceramide diversity. Besides, the most important thing of this annotation system is that the annotation results are organized and visualized intelligibly so that users can interpret the distribution of GSL structures in the MS data rapidly and easily. We will present our first version of the DANGO software and expect to find collaboration partners interested in testing our software and helping us to improve the system and make it more user friendly.

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### (136) Leucine-rich repeat-containing G-protein coupled receptor 6 (LGR6) functions in the O-GlcNAc-mediated regulation of colon cancer stem cell driven tumorigenesis

Huabei Guo[1,2], Alison V. Nairn[1,2], Shutan Xu[2], Tamas Nagy[3], Shaying Zhao[2], Kelley W. Moremen[1,2], Phillip Buckhaults[4] and Michael Pierce[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]Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA,30602; [4]South Carolina College of Pharmacy, The University of South Carolina, Columbia, SC 29208*

We have focused on the *O*-GlcNAc-mediated epigenetic regulation of human colon cancer cells and cancer stem cells (CCSC). Xenograft tumors from colon tumor cells with the knockdown of OGT (*O*-linked *N*-acetylglucosamine transferase) grew significantly slower than those formed from control cells, indicating reduced proliferation of tumor cells due to inhibition of OGT expression. A significant reduction of CCSC population was observed in these tumor cells after OGT knockdown, while tumor cells treated with an *O*-GlcNAcase inhibitor showed an increased CCSC population, indicating that O-GlcNAc levels regulated the CCSC compartment. When grown in suspension, tumor cells with OGT knockdown showed a reduced ability to form tumorspheres, indicating a reduced self-renewal of CCSC due to reduced levels of O-GlcNAc. To study mechanisms involved, gene expression profiles were determined by using RNA-seq analysis. Many transcripts (301) were differentially expressed (fold-change>1.5 and $p < 0.05$) between control and OGT knockdown cells. The LGR6, a member of the leucine-rich repeat-containing G protein-coupled receptor superfamily and one of genes mutated in colon cancer, was observed to be increased by almost 6-fold in expression in tumor cells with OGT knockdown, while the expression of other family members (LGR4 and 5) were unaffected. Increased LGR6 expression after OGT knockdown was further validated by PCR experiments. A large GC rich region (CpG island) near the TSS site of the LGR6 gene was identified, and high methylation of this region was observed in different colon tumor cell lines by MSP-PCR. Moreover, analysis of gene expression and methylation using the TCGA database for 450 cases of colorectal adenocarcinomas revealed significantly lower gene expression and higher levels of overall gene methylation of LGR6, compared to LGR4 and 5. These results strongly implicate that LGR6 may be involved, functioning as a tumor suppressor, in the regulation of CCSC and colon tumorigenesis mediated by O-GlcNAc expression levels. Experiments are in progress to validate the possible tumor suppressor functions of LGR6 in vivo and in vitro.

### (137) T Cells Require Extended O-Glycans To Populate Peripheral Lymphoid Organs

Christopher E. Cutler[1,2] and Richard D. Cummings[1]

*[1]Beth Israel Deaconess Medical Center; [2]Emory University*

T cells depend on a wide variety of glycoproteins for adhesion, signal transduction, and effector functions. With a few exceptions, the role of glycosylation in those functions remains uncharacterized. Similarly, while global glycosylation of T cells is dynamic and changes with maturation and activation, the role glycosylation plays in T cell development and function is poorly understood. To explore this role, we generated mice whose T cells lack the essential chaperone Cosmc, without which cells cannot create extended O-glycans. These mice had dramatically reduced numbers of T cells in both blood and peripheral lymphoid organs, and the remaining T cell population was dominated by cells that had escaped gene deletion. In contrast, thymic populations were undiminished; knockout cells were present and appeared to develop normally. Adoptive transfer of mature thymocytes showed that knockout T cells were unable to populate peripheral organs and were rapidly cleared from circulation. These results demonstrate that extended O-glycosylation of T cells is crucial to their ability to migrate into lymphoid organs and populate the periphery.

### (138) Characterization of a novel hemolysin that possesses specificities for glycoproteins and lipids

Christina Welch, Priyanka Kadav, Ni Fan, Robert Brown, Alexander Vizurraga, Purnima Bandyopadhyay and Tarun Dam

*Mechanistic Glycobiology, Department of Chemistry, Michigan Technological University*

We have isolated a natural hemolytic agent named Hemolysin X or HelyX from closely related plant species. HelyX is present in pre-and post-germination stages of those plants. When tested with human and rabbit erythrocytes, HelyX showed vigorous lytic activity. HelyX occurs in association with an agglutinin and they could be separated from each other by size exclusion chromatography. Among the ligands tested, thyroglobulin, asialofetuin and fibrinogen inhibited the activity of HelyX as well as the agglutinin. In addition, HelyX also recognized certain lipid structures such as desmosterol, cholesterol, progesterone, stigmasterol and palmitic acid. The kinetics of lytic activity and its inhibition by ligands were rapid. Activity of HelyX was completely inhibited by isolated erythrocyte membranes. This observation suggests that the hemolysin interacts with the plasma membranes of intact erythrocytes before rupturing them. Electron microscopic studies indeed revealed that interaction of HelyX with erythrocytes ledto the formation of pores on the cell surface before completely distorting the membrane structure. In vitro experiments showed that HelyX was able to dissolve preformed blood clots. Because of its ability to dissolve blood clots and its high affinity interactions with fibrinogen, cholesterol, desmosterol and progesterone, HelyX could potentially be used as a valuable tool for biochemical and clinical research.

### (139) Microbiota polysaccharides mediate immune suppression via memory T to regulatory T cell cooperativity

Mark B. Jones, Jenny L. Johnson, Carlos A. Alvarez, Julie Y. Zhou, Kelsey D. Oliva, Nathan Morris and Brian A. Cobb
*Case Western Reserve University*

Beneficial gastrointestinal microbiota shape the development of the immune system and can steer overall immunologic bias away from autoimmunity toward homeostasis. To induce this response, commensal bacterial products either elicit potent inflammatory responses or educate the adaptive immune system resulting in restraint of excessive immune responses. Polysaccharide A (PSA) from *Bacteroides fragilis* is a well-known glycoantigen shown to suppress inflammation in a T cell dependent fashion. Memory T cells mediate rapid responses upon re-exposure to an antigen whereas regulatory T cells (Treg) play key roles in suppressing immune responses and preventing autoimmunity, typically through the secretion of immunomodulatory cytokines like IL-10. Our laboratory has shown that exposure to PSA expands a population of antigen experienced FoxP3$^-$CD4 + CD45Rb$^{lo}$T$_{EM}$ (Rb$^{lo}$T$_{EM}$) cells which are capable of amplifying IL-10 production in peripheral tissues upon inflammatory stimulus. We have defined the molecular interaction between T cells using contact assays, RNAseq, Luminex, and antibody neutralization analyses to demonstrate that these Rb$^{lo}$T$_{EM}$ cells synergistically induce IL-10 production in Tregs via secretion of cytokine mediators when co-cultured *in vitro*. Moreover, injection of either Rb$^{lo}$T$_{EM}$ cells or their mediating cytokines was sufficient to reverse pulmonary pathology during inflammatory models. These observations lead to a model in which the commensal microflora are linked to peripheral immunomodulation through T cell cooperation between Rb$^{lo}$T$_{EM}$ cells and tissue-resident Tregs. Further investigation on the mechanism of this pathway, where Rb$^{lo}$T$_{EM}$ cells dampen potentially harmful immune reactivation, may provide new therapeutic targets for the treatment of inflammatory disease and autoimmunity.

### (140) Cooperative ligand binding of a C-type lectin like receptor Dectin-1

Yoshiki Yamaguchi[1,2], Hari P. Dulal[1,2], Yoshiyuki Adachi[3] and Naohito Ohno[3]
[1]*Structural Glycobiology Team, RIKEN, Japan;* [2]*Tokyo Medical and Dental University, Japan;* [3]*Tokyo University of Pharmacy and Life Science, Japan*

Recent advances in structural analyses of mammalian lectin receptors reveal the atomic details to attain the fine specificities toward diverse endogenous and exogenous glycans. C-type lectin is one of the largest lectin families in mammals and we are extending the knowledge of ligand binding mechanisms (1). Dectin-1 is a mammalian C-type lectin like receptor and engaged in immunological defense against fungal pathogens. Upon binding with fungal β-glucans, the cytoplasmic tail of Dectin-1 receives phosphorylation and this triggers secretion of certain inflammatory cytokines such as tumor-necrosis factor (TNF) and IL-12. However the mechanism is unknown how the β-glucan binding to Dectin-1 induce such intracellular signaling. To address this question, we first investigated the ligand binding of Dectin-1 in detail. A series of NMR study revealed that β-glucan-Dectin-1 interaction is enhanced upon increasing β-glucan chain length (2). Furthermore, a β-glucan ligand laminarin (degree of polymerization ~ 25) strongly binds to Dectin-1 and induces oligomerization of Dectin-1 (3). Dose-response curve revealed that wild-type CTLD cooperatively binds to laminarin with the Hill coefficient of ~ 3, while some Dectin-1 mutants abolish or decrease the cooperativity with the Hill coefficient. We suggest that the ligand-induced cooperative oligomer formation of Dectin-1 is physiologically relevant in sensing exogenous β-glucan and triggering subsequent intracellular signaling.

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### (141) Plasma glycomics predicts cardiovascular sequelae in patients with controlled HIV infections

Douglas M. Oswald[1], Edward S. Sim[1], Obada Farhan[2], Sara Debanne[2], Nathan Morris[2], Benigno Rodriguez[3], Mark B. Jones[1] and Brian A. Cobb[1]
[1]*Department of Pathology, Case Western Reserve University School of Medicine;* [2]*Department of Population and Quatitative Health Science, Case Western Reserve*

*University School of Medicicne; [3]Department of Medicine, Division of Infectious Diseases and HIV medicine, Case Western Reserve University School of Medicine and University Hospitals Cleveland Medical Center*

Despite effective control of HIV infection with antiretroviral drugs, HIV-infected individuals are beset by high incidences of secondary diseases. These sequelae, such as cardiovascular disease (CVD), are poorly understood and represent a massive health burden for both patient and society. To date, the underlying mechanisms leading to HIV-induced CVD remain unknown, and predictive biomarkers have been elusive. Here, we apply high throughput glycomic analysis to a population of banked HIV-infected human plasma samples to identify glycan biomarkers that might be harnessed to predict cardiovascular events. Patient samples were collected prior to any known cardiac event or CVD diagnosis and later sorted on CVD case and non-CVD control basis. We utilized a newly developed multi-target ELISA-based analysis to build a glycomic fingerprint, which we then correlated with thepatient's development of CVD. Our model identified a seven-lectin glycan signature that was strongly predictive of increased risk for future disease, suggesting that the human plasma glycome can serve as a biomarker for HIV-induced secondary disease. Our findings reveal opportunities to interrogate specific target glycoforms and glycoproteins, their contributions to disease, and potential interventions, thereby enabling risk screening and preventative therapies for HIV-infected patients to significantly reduce their morbidity and mortality.

### (142) Site-specific glycosylation of viral surface proteins using mass spectrometry

Cassandra L. Pegg, Toan K. Phung and Benjamin L. Schulz
*School of Chemistry and Molecular Biosciences, University of Queensland, Queensland 4072, Australia*

The importance of glycosylation in host-pathogen interactions is well established. With respect to viral infection, glycosylation of viral surface proteins has been implicated in protein biosynthesis, viral attachment and entry, induction of immune responses and evasion of host-immune defences. Furthermore, viral glycoproteins can be used as targets for vaccines and antiviral therapies. Glycosylation is specific to various organisms, tissues and cell lines, as such, production of viral proteins in different expression systems can result in substantially different glycosylation profiles. Defining glycosylation of viral surface proteins produced during natural infection or *in vitro* is therefore important for the elucidation of host-virus interactions and for the design of viral therapeutics. Site-specific analyses of occupancy and the monosaccharide composition of glycans from virion-derived proteins can be challenging. Adequate quantities of proteins can be difficult to obtain from natural systemsand in some cases, from virions propagated in relevant cells lines. The work presented herein highlights mass spectrometry-based methods to characterise glycosylation of viral proteins. These glycoproteomic methods may help inform us of the functional roles glycans play on viral proteins and help ensure accurate glycosylation profiles when producing these proteins for therapeutic use.

### (143) Galectins from the eastern oyster (Crassostrea virginica) preferentially recognize the protozoan Perkinsus marinus by carbohydrate-based parasite mimicry

Chiguang Feng[1], Anita Ghosh[2], Mohammed N. Amin[3], Tsvetan R. Bachvaroff[4], Mario A. Bianchet[2], Lai-Xi Wang[3], Daniel Zheng[1,6], Deandra Watson[1,7], Iain B.H. Wilson[5] and Gerardo R. Vasta[1]
*[1]Department of Microbiology and Immunology, University of Maryland School of Medicine, UMB, Institute of Marine and Environmental Technology, Baltimore, Maryland, USA; [2]Departments of Neurology and Biophysics and Biophysical Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; [3]Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland, USA; [4]University of Maryland Center for Environmental Science, and Institute of Marine and Environmental Technology, Baltimore, Maryland, USA; [5]Department für Chemie, Universität für Bodenkultur, Vienna, Austria; [6]Visiting Summer Intern from Centennial High School, HCPS, Maryland; [7]Visiting Summer Intern from Coppin State University, Baltimore, Maryland*

Galectins are highly conserved lectins that are key to multiple biological functions, including pathogen recognition and regulation of immune responses. However, CvGal1, a galectin expressed in phagocytic cells (hemocytes) of the eastern oyster (*Crassostrea virginica*), is "hijacked" by the parasite *Perkinsus marinus* to enter the host, where it causes systemic infection and death. A screening of an oyster hemocyte cDNA library revealed a second galectin (CvGal2) with four tandemly-arrayed carbohydrate recognition domains (CRDs). Although a phylogenetic analysis of the CvGal2 CRDs suggests close relationships with homologous CRDs from CvGal1, a glycan array analysis revealed that CvGal2 has broader binding specificity for ABH blood group oligosaccharides. Further, SPR analysis demonstrated significant differences in the binding kinetics of CvGal1 and CvGal2, and structural modeling revealed substantial differences in their interactions with the oligosaccharide ligands. Both CvGal1 and CvGal2 are expressed in hemocytes, released to the extracellular space, and bind to the hemocyte surface. They also bind to *P. marinus* trophozoites in a dose-dependent and ß-galactoside-specific manner. Strikingly, negligible binding was observed for *P. chesapeaki*, a sympatric parasite species mostly prevalent in the softshell clam *Mya arenaria*. We hypothesize that the differential recognition of *Perkinsus* species by the oyster and clam galectins could be consistent with their relative prevalence in oyster and clam species, and supports the galectins' role in facilitating parasite entry and infectivity by carbohydrate-based

parasite mimicry in a host-preferential manner. Further, we successfully isolated a novel galectin from the softshell clam that we named as MaGal1. MaGal1 strongly cross-reacted with anti-CvGals antibodies, suggesting the presence of shared epitopes between oyster and clam galectins. Glycan array analysis, however, revealed that MaGal1 preferentially binds to Gal(α1-3/4)Gal(ß1-3/4)GlcNAc, unlike both CvGal1 and CvGal2 that prefer ABH blood group oligosaccharides. Current studies are aimed at the detailed structural characterization of CvGal1, CvGal2, and MaGal1, and their differential recognition *Perkinsus* species. [Supported by grants IOS-0822257, IOS-1063729, and IOS-1656720 from NSF, and grant 5R01GM070589-06from NIH to GRV; and grant R01 GM080374 from NIH to LXW. We are grateful to Dr. Richard D. Cummings, Dr. David Smith and Dr. Jamie Heimburg-Molinaro, Core H-CFG and NCFG, for glycan array analysis].

### (144) Probing the Function of N-glycans in Platelet-Collagen Interaction

Christian Toonstra and Hui Zhang
*Department of Pathology, Johns Hopkins University*

Platelets are crucial mediators of primary hemostasis and endothelial repair, however, chronic pathological platelet activation also plays a critical proximal role in progressive vascular occlusion diseases. Collagen is a potent agonist for a number of platelet glycoproteins, presenting itself as a potential key contributor to platelet function. The fine-details that dictate the interactions between collagen and collagen-binding proteins from platelets are poorly understood, in particular, glycosylation could be a key determinant in collagen binding. Deciphering the specific impact of glycosylation in modulating protein-protein interactions is challenging, particularly within the context of the complex cellular milieu. In this study, the analyses of N-glycosylation on platelet glycoproteins and the specific roles of N-linked glycans from glycoproteins in collagen binding was explored. We developed a technique using a combination of collagen affinity chromatography and LC-MS/MS analysis to measure protein-glycoprotein interactions with a key focus on determining the impact of glycan manipulation on protein-protein interactions via treatment with either peptide-N-glycosidase F (PNGase F) or α2-3, 6, 8-Neuraminidase. The approach was validated in three modules, including a lectin-glycoprotein proof-of-concept system, a model system comprised of isolated plasma fibronectin binding to collagen, and a complex system following the binding of fibronectin to collagen within platelet lysate. The identities of proteins interacting with collagen were determined using LC/MS/MS analysis incorporating both label-free and tandem mass tag (TMT) labelling, providing a quantitative assessment of the impact of glycosylation on collagen affinity. Specific glycopeptide contributors to collagen binding were identified by glycopeptide enrichment using anion-exchange chromatography followed by intact glycopeptide analysis using higher-energy collisional dissociation (HCD) fragmentation. Preliminary results have demonstrated the importance of fibronectin glycosylation for optimal binding, suggesting a critical functional role of N-glycans in facilitating collagen binding. The strategy described provides a robust method to study protein-glycoprotein interactions in complex matrices that has the potential to be applied to a broad number of applications. In addition, the described method contributes to our understanding of the fundamental requirements for optimal platelet-collagen binding, and may provide insight into the development of more targeted anti-thrombic agents.

### (146) Kdn, a Free Sialic Acid in Humans has Therapeutic Potential Against Non-typeable Haemophilus influenzae (NTHi) infections

Sudeshna Saha[1], Sandra Diaz[1], Biswa Choudhury[1], Victor Nizet[2], Sanjay Ram[3], Ian Schoenhofen[4] and Ajit Varki[1]
*[1]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, USA; [2]Department of Pediatrics, and the Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA, USA; [3]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts, MA, USA; [4]Human Health Therapeutics Portfolio, National Research Council of Canada, Ottawa, Ontario, Canada*

The Gram-negative coccobacillus *Haemophilus influenzae* is a common human-specific mucosal commensal and opportunistic infectious pathogen. While vaccination against the encapsulated form is efficacious, absence of a vaccine against the non-encapsulated ("nontypeable") form and its steadily rising antibiotic resistance is emerging as a global health problem. Non-typeable *Haemophilus influenzae* (NTHi) can cause respiratory tract infections including otitis media in children and sinusitis or acute exacerbations of chronic obstructive pulmonary disease (COPD) in adults. The NTHi lipooligosaccharide (LOS) can be "cloaked" by a terminal 9-carbon monosaccharide called *N*-acetylneuraminic acid (Neu5Ac), acquired by the bacterium via highly efficient uptake from host body fluids. This strategy blunts alternate pathway complement activation/killing and masks antibody recognition of underlying glycoconjugate antigens, while facilitating colonization and biofilm formation. Neu5Ac belongs to a large, diverse family of nonulosonic acids among which Neu5Ac, N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn) are present in vertebrates and called sialic acids. We have recently shown that the replacement of surface Neu5Ac with sialic acid analogs assists killing and clearance of multidrug-resistant gonococcal infection (1). To look for similar therapeutic interventions against NTHi, we exposed this organism to free Kdn in place of free Neu5Ac. While Kdn did not affect overall growth of NTHi, HPLC analysis of the bacterial pellet shows accumulation of glycosidically-bound Kdn and likely incorporation into the bacterial LOS. However, unlike the protection

provided by Neu5Ac incorporation, growth in presence of Kdn resulted in a significant increase of NTHi killing by normal human serum. Currently, we are working to understand the effects of Kdn incorporation in NTHi on different aspects of the immune response, with a view to eventually developing it as a potentially non-toxic therapeutic agent. Considering the wide abundance of sialic acid uptake and metabolism-related genes among microorganisms including several human pathogens, this approach may also be worth exploring with other pathogenic microbes that are known to efficiently take up free Neu5Ac from body fluids.

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## (147) Hydrogen bonding and three-dimensional structure in glycans from bacteria and cancer

Marcos D. Battistel, Hugo F. Azurmendi and Daron I. Freedberg
*CBER/FDA*

Intramolecular hydrogen bonds (Hbonds) are important stabilizing forces in biomolecules. They are well established in aqueous solutions of proteins and nucleic acids, however Hbonds are not routinely characterized in aqueous solutions of glycans. Their direct detection would greatly improve our ability to determine glycan three-dimensional structure. Here we report direct detection of intramolecular Hbonds in glycans by NMR spectroscopy. In NMR structural studies, we utilized fully $^{15}N$,$^{13}C$ labeled glycans to directly and indirectly detect intramolecular Hbonds arising from NH groups. These led us to identify a helical motif in α-2,8 sialic acid oligomers. We also recently detected glycan hydroxyl (OH) groups in aqueous samples and used the OH signals for OH Hbond direct detection, including an OH-based Hbond between two sialic acid residues in a dimer of α-2,8 sialic acid. However, in many cases OH groups compete with water-based Hbonds, and are transient, which interferes with their direct detection. Therefore, we developed new NMR methods to infer these Hbonds from reduced OH/OD exchange rate constants. This is significant as the OH groups can now be used as NMR handles and structural probes to expand the repertoire of tools for NMR structure-function studies on glycans. Finally, we were able to detect the presence of CH-based Hbonds and to show their placement in sialyl Lewis-X. In this presentation, we highlight various types of Hbonds in glycans and the three-dimensional motifs that can be gleaned from their detection. These experimental measurements will help shed light on glycan-glycan interaction and glycan recognition by proteins, which can improve our understanding of Hbond functionality in cancer and bacterial polysaccharide vaccines.

## (148) Identifying O-acetylated Sialic Acids Using Viral-Derived Sialoglycan-Recognizing Probes

Brian R. Wasik, Karen N. Barnard, Brynn K. Lawrence and Colin R. Parrish
*Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA*

Sialic acids (Sias) posses a unique chemistry allowing for vast modified forms being found in nature. The relative display and distribution of sialoglycans with Sia modifications in varied organisms is fairly unknown due to limited molecular tools. We are using recombinant sialoglycan recognizing probes (SGRPs) generated from viral proteins for detection of specific Sia forms. Nidovirus HEs are specific for O-acetyl (O-Ac) Sia modifications: MHV-S for 4-O-Ac, BCoV-Mebus for 7,9-O-Ac, PToV-P4 for 9-O-Ac. The viral HE glycoprotein is fused to the Fc region of human IgG1, and the receptor-binding and specific acetylesterase activity of the proteins can be ablated with an active-site mutations. Viral SGRPs can be used in a variety of applications, including: glycan microarray, ELISA, Western Blot and FACS using cell lines. Our framework for validation of the SGRPs and investigation of the biological role of O-acetylated Sia forms is in the context of influenza viruses. Modified Sias have been identified as influenza infection inhibitors (horse or guinea pig serum, Neu4,5$Ac_2$) or as negative regulators on NA efficiency (Neu5,9$Ac_2$). Influenza C and D strains utilize 9-O-Ac modified Sia as primary receptors. Therefore, modified Sias may contribute to evolved Hemagglutinin (HA) and Neuraminidase (NA) specificities seen between viral strains and different hosts. We have used SGRPs to perform *in situ* histological surveys of O-Ac modified Sias in tissues of multiple influenza hosts, including mouse, human, ferret, guinea pig, pig, horse, dog, duck, and embryonated chicken eggs. Our survey confirms the presence of O-acetyl modified Sias in mammalian respiratory and avian tissues with varied patterns of distribution. The 4-O-Ac Sia is significantly enriched in horse and guinea pig tissues, but can be detected in other hosts, including in mice and avian respiratory (duck). The 9- and 7,9-O-Ac Sias are more broadly distributed across host species and tissues, being particularly enriched in human and mice tissues, as well as extra-embryonic chicken egg membranes. Confirming the presence of modified Sias in target influenza host tissues justifies the need for experimental systems to understand their effects. Having identified modified Sias in major cell culture lines used for influenza studies, we have generated cell lines (HEK293, A549, MDCK) varied in 9-O-Ac Sias displayed. These cell lines were developed using CRISPR-Cas9 gene editing to remove the 9-O-acetyltransferase activity or by overexpression of the enzyme via a stable expression plasmid. These cell lines are part of a new genetic approach to generating 'glyco-engineered' experimental systems.

**(149) Intracellular galectins control cellular responses commensurate with cell surface carbohydrate alterations**

Ming-Hsiang Hong[1], Wei-Han Lin[1], I-Chun Weng[1], Yu-Hsien Hung[2], Hung-Lin Chen[1], Huan-Yuan Chen[1], Wei-Yuan Yang[2] and Fu-Tong Liu[1]

*[1]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China (R.O.C); [2]Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, Republic of China (R.O.C)*

Galectins are animal lectins found in the cytosol with individual preferences for an array of beta-galactose-containing oligosaccharides. They are recruited to intracellular vesicles that are damaged following various insults. We applied a light-induced endocytic vesicle-impairing regimen to study this process. The endocytosed amphiphilic photosensitizer AlPcS2a (disulfonated aluminum phthalocyanine) was illuminated with red light. We found that internalized cell surface glycoconjugates in endocytic vesicles mediate intracellular galectin accumulation when the vesicles are damaged in Chinese hamster ovary (CHO) cells. We used different glycosylation-mutant CHO cell lines, and found that the amount of accumulated galectins was dependent on the composition and amount of carbohydrates recognized by the protein. Importantly, galectins could detect cell surface carbohydrate alterations, resulting in different amounts of galectins accumulating around damaged endocytic vesicles. Specifically, the amounts of galectin-3 and -8 accumulated in damaged endocytic vesicles were significantly higher and lower, respectively, when sialic acids were removed from the cell surface. Furthermore, the accumulated galectins could direct distinct autophagy adaptor proteins to impaired endocytic vesicles, which was also significantly affected when sialyation of the cell surface glycans was altered. We conclude that galectins govern cellular responses associated with endosomal damage in a manner that reflects dynamic cell surface glycan modifications.

**(150) Structural Insights into Bacterial Sialic Acid Transport**

Weixiao Y. Wahlgren[1,2], Elin Dunevall[1], Rachel A. North[3,8], Aviv Paz[4], Mariafrancesca Scalise[6], Paola Bisignano[5], Johan Bengtsson-Palme[10,2], Parveen Goyal[1,2], Elin Claesson[1], Ulf Nilsson[11], Anne Farewell[1,2], Lorena Pochini[6], Cesare Indiveri[6], Michael Grabe[5], Renwick CJ Dobson[3,9], Jeff Abramson[4,7], S. Ramaswamy[7] and Rosmarie Friemann[1,2]

*[1]Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden; [2]Centre for Antibiotic Resistance Research, CARe, University of Gothenburg, Sweden; [3]Biomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, Christchurch, New Zealand; [4]Department of Physiology, David Geffen School of Medicine, University of California, Los Angeles, United States; [5]Cardiovascular Research Institute, Department of Pharmaceutical Chemistry, University of California, San Francisco, United States; [6]Department DiBEST (Biologia, Ecologia, Scienze della Terra) Unit of Biochemistry and Molecular Biotechnology, University of Calabria, Rende, Italy; [7]Institute for Stem Cell Biology and Regenerative Medicine, Bengaluru, India; [8]Biomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, New Zealand; [9]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne; [10]Department of Infectious Diseases, Institute for Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden; [11]Centre for Analysis and Synthesis, Department of Chemistry, Lund University, Sweden*

Sialic acids comprise a varied group of nine-carbon amino sugars widely distributed among mammals and higher metazoans. Commensal and pathogenic bacteria that colonise heavily sialiated niches (e.g. the mammalian respiratory tract and gut) can scavenge sialic acids from their surrounding environment. Scavenged sialic acid is used as a carbon, nitrogen and energy source, or to evade the host immune response by decorating their outer surfaces in sialic acid.

Bacterial sialic acid membrane protein transport systems have been identified that belong to the tripartite ATP-independent periplasmic transporters, ATP-binding cassette, major facilitator superfamily and sodium solute symporter transport systems.

Here we report the 1.95 Å resolution crystal structure of a specific sialic acid sodium solute symporter, SiaT, in its outward-open conformation. The structure of SiaT was determined in complex with sodium and sialic acid bound, providing insight into how this transporter mediates the movement of sialic acid across the membrane. The overall structure contains 13 transmembrane helices and adopts a LeuT-like fold where the structural core is formed from two inverted repeats of five transmembrane helices each. This type of fold is seen in other sodium symporters, including the leucine transporter (LeuT) and the galactose transporter (vSGLT).

A sodium molecule occupies a binding site equivalent to the Na2-site in other sodium solute symporters. A new and putative Na3-sodium binding site was also found at a previously proposed sodium escape pathway on the intracellular side. Structural and biochemical analyses elucidate essential transport residues, and for the first time a bacterial sialic acid transporter has been characterized. In addition, molecular modeling and molecular dynamics simulations provide insight into the transport mechanism employed by SiaT.

**(151) ST3Gal3 deficient mice exhibit spontaneous and induced morbidity and mortality**

Adrianne L. Stefanski[1], Rachel L. Waagmeester[1], Vanessa L. Richardson[1], Stacey Thomas[2], Dorota S. Raclawska[1], William J. Janssen[1,2] and Christopher M. Evans[1]

*[1]Department of Medicine, University of Colorado Denver, Aurora, CO; [2]Department of Medicine, National Jewish Health, Denver, CO*

The mucin glycoprotein Muc5b is required for normal airway defenses. Muc5b absence in genetically deficient mice is associated with both impaired mucociliary clearance and an accumulation of airspace macrophages (AMs). Muc5b is heavily and selectively α2,3-sialylated in mouse airways and thus provides an endogenous ligand for Siglec-F, a transmembrane immunoinhibitory lectin exclusively expressed in the healthy lung by AMs. Siglec-F has a well characterized binding preference for α2,3 sialylated ligands, and the enzyme ST3Gal3 is required for Siglec-F interaction with airway ligands. The functional consequences of loss of α2,3 sialosides on Muc5b in the context of normal Muc5b apoprotein expression are currently unknown. We used ST3Gal3 deficient mice (*St3gal3*$^{-/-}$) to model a Muc5b sufficient airway lacking the α2,3 sialosides that provide endogenous ligands for Siglec-F on AMs. Importantly, no difference in Muc5b abundance in lung lavage fluid was observed between *St3gal3*$^{-/-}$ mice and wild type (WT) littermate controls. *St3gal3*$^{-/-}$ mice develop spontaneous morbidity and mortality with many untreated *St3gal3*$^{-/-}$ mice dying by or before 24 weeks of age. At baseline, *St3gal3*$^{-/-}$ associated morbidities included lung inflammation, generalized hemorrhage, and unexplained weight loss. In one spontaneously moribund mouse, we observed increased AMs in whole lung lavage compared to a co-housed littermate control. Additionally, heterozygous crosses of *St3gal3*$^{+/-}$ mice result in significantly fewer *St3gal3*$^{-/-}$ offspring than expected (13.6% observed vs. 25% expected, $n = 637$, $p < 0.001$). Currently, we are undertaking a prospective cohort study to fully characterize the natural life course and extent of defects in *St3gal3*$^{-/-}$ micecompared to WT controls. To investigate potential alterations in acute lung inflammation at early time points, WT and *St3gal3*$^{-/-}$ mice were given low-dose (2 μg) lipopolysaccharide (LPS) via intratracheal (i.t.) instillation and airspace cells were harvested 6 hours post-LPS by whole lung lavage. In *St3gal3*$^{-/-}$ mice compared to WT, no differences were observed in the overall numbers or types of immune cells. However, in the resident AM subpopulation we observed a trend ($p = 0.052$) toward decreased Siglec-F levels on cells from ST3Gal3$^{-/-}$ mice ($n = 5$) compared to WT ($n = 6$). Next, to assess severe persistent inflammation, we challenged *St3gal3*$^{-/-}$ ($n = 10$) and WT ($n = 11$) mice with 200 μg LPS i.t. In this inflammatory setting, *St3gal3*$^{-/-}$ mice had significantly increased mortality ($n = 10$, $p = 0.0002$), with a median survival of only 3 days, while the majority (10 of 11) of WT mice recovered normally. Together, these data support a critical role in for *St3gal3*in homeostasis and in acute lung injury. Ongoing studies seek to determine potential links between α2,3 sialosides of Muc5b and Siglec-F expression of AMs.

### (152) Glycoproteomics analysis to examine the role of chlamydial protease-like activity factor

Julian Saba[1], Fred Zinnel[2], Christa Feasley[3], Stuart McCorrister[4], Garrett Westmacott[4], Grant McClarty[4] and Chris Grant[4]

*[1]Thermo Fisher Scientific, Mississauga, ON, Canada; [2]Thermo Fisher Scientific, Somerset, NJ; [3]Thermo Fisher Scientific, West Palm Beach, FL, USA; [4]National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada*

**Background:** Recently proteomics studies have been performed to examine the role of chlamydial protease-like activity factor (CPAF), a secreted virulence factor and its role in immune evasion. However, experiments thus far have ignored glycopeptides that are prevalent in the sample as large scale intact glycopeptide analysis remains challenging by mass spectrometry. Here, we perform large scale intact glycopeptide analysis to examine the role of CPAF targets and derive additional insights from glycoproteomics experiments.

**Method:** Hela 229 cells were infected with *Chlamydia trachomatis* L2 RST5 CPAF-sufficient strain and RST17 CPAF-deficient strain. Proteins were extracted, trypsin digested and enriched for glycopeptides. Samples were analyzed on Orbitrap Fusion Lumos MS. Data analysis was performed using Proteome Discoverer with Byonic node.

**Preliminary Data:** In proteomics experiment ~40–50% of MS/MS spectra are identified. Thus, researchers have focused on developing software algorithms to sequence remainder of the spectra with the hypothesis that these are indeed identifiable spectra from conventional peptides. This assumption ignores the fact that the spectra are the result of PTMs. We have observed some of these unidentified spectra are from glycopeptides. Unfortunately, conventional fragmentations are not ideal for glycopeptide sequencing. Further exasperating the issue is that these glycopeptides are present in low abundance. In our proteomics experiments we observed that 10% of our MS/MS spectra were from glycopeptides. XICs of *m/z* 204.087 (HexNaAc oxonium ion) were performed on LC-MS/MS runs. As precursor ions containing the *m/z* 204.087 could not be sequenced, these were targeted in a glycoproteomics experiment. Overall we identified over 3000 unique intact glycopeptides in a single LC-MS/MS run and over 5000 in triplicate runs, translating into over 600 unique glycoproteins.

**Conclusion:** To our knowledge this is by far the largest number of intact glycopeptides reported in a single experiment.

### (153) Cholera toxin binds to LewisX and fucosylated glycoproteins play a functional role in human intestinal cell intoxication

Jakob Cervin[1], Amberlyn M. Wands[2], Anna Casselbrant[3], Aleksander Cvjetkovic[1], Johanna Estelius[1], Benjamin Dedic[4], Anirudh Sethi[2], Kerri-Lee Wallom[5], Rebecca Riise[6], Malin Bäckström[7], Ville Wallenius[3], Frances M. Platt[5], Michael Lebens[1], Susann Teneberg[4], Lars Fändriks[3], Jennifer J. Kohler[2] and Ulf Yrlid[1]

[1]*Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg;* [2]*Department of Medical Biochemistry, University of Texas Southwestern Medical Center;* [3]*Department of Gastrosurgical Research and Education, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg;* [4]*Department of Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg;* [5]*Department of Pharmacology, University of Oxford;* [6]*Sahlgrenska Cancer Center, University of Gothenburg;* [7]*Mammalian Protein Expression Core Facility, University of Gothenburg*

Cholera toxin (CT) enters and intoxicates host cells after binding cell surface receptors via its B subunit (CTB). We have recently shown that in addition to the previously described ganglioside GM1, CTB binds to fucosylated proteins on cells from human colon. Here we report that CT binds the Lewis$^X$ glycan *in vitro* when this moiety is linked to proteins but not to ceramides and that this binding is dependent on the fucose in Lewis$^X$. Analyzing primary cells from human small intestine and granulocytes with flow cytometry, we show that CTB binding correlates with Lewis$^X$ expression. Furthermore, we found that interference with fucosylated moieties blocks binding and subsequent CT-driven ion secretion by primary human jejunal biopsies in Ussing chambers. Finally, we show that knockout mice, lacking GM1 and GM1-related glycosphingolipids, still present an intact diarrheal response when gavaged with CT *in vivo* supporting the presence of functional receptors other than GM1. These findings open up new avenues for therapies to block CT action and for design of detoxified enterotoxin-based adjuvants.

### (154) Accelerated Aging and Turnover of Host Anti-Inflammatory Enzymes in the Pathogenesis of Gram-negative Sepsis

Won Ho Yang[1,2], Douglas M. Heithoff[1,3], Peter V. Aziz[1,2], Benjamin S. Haslund-Gourley[1,2], Michael J. Mahan[1,3], Victor Nizet[4] and Jamey D. Marth[1,2,3]

[1]*Center for Nanomedicine;* [2]*Sanford-Burnham-Prebys Medical Discovery Institute;* [3]*Department of Molecular, Cellular, and Developmental Biology, University of California-Santa Barbara, Santa Barbara, California 93106;* [4]*Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California 92093*

Recent discoveries by this laboratory identified a mechanism of secreted protein aging and turnover that is composed of glycosidase–mediated N-glycan remodeling (Yang et al., 2015). We now find that this mechanism is modulated in mouse models of sepsis caused by infection-with the human bacterial pathogens *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, and moreover differently from sepsis caused by the Gram-positive pathogens *Streptococcus pneumoniae* and *Staphylococcal aureus*. During the onset of sepsis, we have measured an increased rate of N-glycan remodeling with ensuing endocytic lectin ligand formation among secreted proteins in the blood plasma, resulting in rapid decreases in the abundance and function of two host anti-inflammatory enzymes, namely tissue non-specific and intestinal alkaline phosphatases (TNAP and IAP). Our findings demonstrate that both of these secreted glycoprotein enzymes are post-translationally regulated by sialylation as a means of determining their concentrations and activities in the blood. Our data further reveal that the endogenous sialyltransferase ST3Gal6 is responsible for TNAP and IAP sialylation, without which both alkaline phosphatase isozymes are rapidly cleared from circulation by the Ashwell-Morell receptor (AMR). This accelerated remodeling of TNAP and IAP in sepsis is due to the induction of host neuraminidase activity in the blood. This induction includes Neu1 and Neu3 and can be recapitulated by the lipopolysaccharide component of Gram-negative bacteria, thereby accelerating the aging and turnover of the anti-inflammatory enzymes TNAP and IAP. We further show that the induction of Neu activity with the subsequent reduction of alkaline phosphatase activity are responsible for increased LPS-phosphate levels linked with inflammation and reduced survival of Gram-negative sepsis. These findings demonstrate unexpected features of pathogen and host interactions during sepsis that target an intrinsic host mechanism of secreted protein aging and turnover. The resulting rapid changes in protein abundance and function among the secreted proteome have significant impacts on the pathogenesis and survival of sepsis.

### (155) Surface Glycan-Binding Protein Uniquely Facilitate Starch Metabolism in the Gut Symbiont Bacteroides thetaiotaomicron

Matthew Foley, Hannah Tuson, Julie Biteen and Nicole Koropatkin

*University of Michigan*

The human gut microbiota plays an essential role in maintaining optimal health, yet little is known about how many of these organisms harvest nutrients from their environment. Dietary glycans are a controllable variable that shape the composition of the human gut microbiota, and microorganisms that colonize this niche have strategies to efficiently compete for these nutrients. A dominant phylum of the gut, the Gram-negative Bacteroidetes, can utilize numerous dietary and host-derived glycans. Many gut Bacteroidetes devote significant portions of their genome towards polysaccharide utilization loci (PULs) that each encode several proteins that bind, degrade, and import different sugars. Each PUL is individually regulated and encodes for proteins that target a unique glycan structure. Conserved across all known PULs are homologs of the prototypical starch utilization system (Sus) proteins SusC, a TonB-dependent transporter (TBDT), and SusD, a starch binding protein, originally described in the model species Bacteroides

thetaiotaomicron (Bt). In the Sus, SusCDEFG operate on the cell surface to capture, degrade and import starch into the cell. SusC and SusD are both required for Bt growth on starch and interact, although the nature of this interaction and other proteins present in this complex are not described. My data demonstrate that SusE, like SusD, can provide a starch-binding site for glycan transport through SusC, which highlights a new feature of the SusE-like family of proteins within the Bacteroidetes. Furthermore, using quantitative proteomics I demonstrate that SusCDE interact in the membrane, and that the stoichiometric ratio of interacting proteins is affected by formaldehyde crosslinking. In hopes of understanding the relationship between the SusCDE complex and the remaining proteins SusFG, I employ single molecule imaging to visualize SusE, SusF, and SusG dynamics in the membrane of live cells. Imaging shows that, unlike the highly mobile SusG, SusE and SusF are static, although the reason for this is unclear. The goal of my research is to understand how these Sus protein interactions facilitate glycan utilization. Because homologs of these proteins are central to all PULs, understanding how they work together to import starch will inform a general model of the Sus-like paradigm used by the Bacteroidetes for glycan uptake.

### (156) Recurrent Infection Progressively Disables Host Protection Against Intestinal Inflammation

Won Ho Yang[1,2,3], Douglas M. Heithoff[1,3], Peter V. Aziz[1,2,3], Markus Sperandio[4], Victor Nizet[5], Michael J. Mahan[1,3] and Jamey D. Marth[1,2,3]

*[1]Center for Nanomedicine; [2]SBP Medical Discovery Institute; [3]Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, California 93106; [4]Walter Brendel Center for Experimental Medicine, Ludwig-Maximilians-University, Munich, Germany; [5]Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California 92093*

Inflammation of the intestinal tract is the defining feature of colitis and the human inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis. These syndromes appear to arise primarily from unidentified environmental factors. Among human monozygotic twins for example, disease concordance is approximately 30% in Crohn's disease and less than 20% in UC. Pathogenic infection has been studied as an environmental factor precipitating intestinal inflammation, and bacterial infections are linked to seasonal increases in hospital admissions involving intestinal inflammation and IBD. We have observed that recurrent low-titer ($2 \times 10^3$ cfu) non-lethal gastric infections of Gram-negative *Salmonella enterica* Typhimurium (*ST*), a major source of human food poisoning, caused inflammation of murine intestinal tissue, predominantly the colon, which persisted following pathogen clearance and escalated in severity with repeated infections. *ST* infection disabled a host mechanism of protection by inducing intestinal neuraminidase activity and thereby accelerating the molecular aging and clearance of host intestinal alkaline phosphatase (IAP). Disease onset and escalation were linked to IAP desialylation and endocytic degradation, and were recapitulated by a genetic deficiency of IAP sialylation. Pathogenesis ensued by a Tlr4-dependent mechanism responding to infection and amplifying the abundance of LPS-phosphate in the colon. Oral administration of IAP or the marketed anti-viral neuraminidase inhibitor Zanamivir were similarly therapeutic with reductions of LPS-phosphate and intestinal inflammation.

### (158) Regulation of endothelial N-glycans in atherosclerosis: a role for alpha -mannosidases

Kellie Regal-McDonald[1,2], Jarrod W. Barnes[3] and Rakesh P. Patel[1,2]

*[1]Department of Pathology; [2]Center for Free Radical Biology; [3]Department of Pulmonary, Allergy, and Critical Care Medicine, University of Alabama at Birmingham, Birmingham, AL*

Enhanced monocyte adhesion and migration into the sub-endothelial compartment of the vessel wall is a hallmark of atherosclerotic plaque development. Monocyte interactions with endothelial cells (ECs) are mediated by glycosylated surface adhesion proteins, such as intracellular adhesion molecule 1 (ICAM-1). While these proteins are typically expressed with only complex-type N-glycans on the cell surface, we have previously shown that a high-mannose (HM) N-glycoform is also expressed during inflammation. Rolling and adhesion of CD16+ monocytes to HM-ICAM-1 is greater compared to the complex ICAM-1 glycoforms, leading to the hypothesis that HM-ICAM-1 is the primary glycoform of ICAM-1 that mediates monocyte recruitment to the vessel wall during atherogenesis. In this study, we first utilized a proximity-ligation assay (PLA), to test if HM epitopes co-localize with ICAM-1 in: 1- human atherosclerotic lesions, 2- chronic kidney disease patients with fistulas, 3- lesions from pulmonary arterial hypertension, and 4- cultured ECs stimulated with TNF-alpha. PLA staining showed that HM-ICAM-1 is present in stage 3 lesions and endothelial dysfunction associated with fistulas. However, no HM-ICAM-1 was detected in the damaged pulmonary hypertensive endothelium, suggesting specificity for the lesions in non-pulmonary vascular tissues. In cultured EC, HM-ICAM1 was formed early and temporally, followed by the formation of complex ICAM-1. To address how HM-ICAM-1 is regulated, we have focused on the alpha-mannosidases; a class of enzymes responsible for the early processing steps of N-glycosylation in the secretory pathway. We have found that alpha-mannosidase activity (class I) is inhibited early (within 4 h) after TNF-alpha stimulation, independent of NF-kB activation.

These data establish a novel glycan-mediated mechanism for monocyte-endothelial adhesion facilitated by HM epitopes on ICAM-1, and identify endothelial alpha-mannosidases as potential therapeutic targets.

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### (159) The zebrafish tandem-repeat galectin 9 (Drgal9-L1) promotes in vitro adhesion and infection of the infectious hematopoietic necrosis virus (IHNV)

Kelsey Abernathy[1], Justin Mancini[1], Nuria González-Montalbán[1], Chiguang Feng[1], Sheng Wang[2], Lia Schipper[3] and Gerardo Vasta [1]

*[1]Department of Microbiology and Immunology, University of Maryland School of Medicine, UMB, Institute of Marine Environmental Technology, Baltimore, Maryland; [2]School of Life Sciences, Sun Yat-sen University, Guangzhou, PR China; [3]North County High School, Glen Burnie, MD*

Galectins are a structurally and evolutionarily conserved class of β-galactoside-binding lectins characterized by the presence of a unique sequence motif in their carbohydrate recognition domains (CRDs). Initially expressed in the cytosol and secreted into the extracellular space where they bind soluble, membrane, and matrix associated glycoconjugates, they are classified into three types, proto, chimera, and tandem repeat, based on the organization of their CRDs. Galectins are involved with early developmental processes and immune regulation through binding to endogenous ligands, and more recently in innate immunity by functioning as pattern recognition receptors (PRR). Previous work in our lab revealed that tandem repeat recombinant galectin-9 (Drgal9-L1) cloned from the zebrafish (*Danio rerio*) interacts directly with the glycoprotein of the infectious hematopoietic necrosis virus (IHNV) to promote viral adhesion to the fish epithelial cell surface. Based on our observations we hypothesized that Drgal9-L1 can crosslink the virion to the epithelial cell surface by binding glycans on the cell and viral surface, thereby enhancing viral adhesion and modulating viral infectivity. To test this hypothesis and determine any potential binding preference of the Drgal9-L1's CRDs (N-terminal and C-terminal), two different approaches were used: CRD inactivation via mutation and enzymatic cleavage at the peptide linker. Glycan array analysis of whole Drgal9-L1, C-terminal CRD, N-terminal CRD, and C-terminal mutant Drgal9-L1 indicated a strong binding preference for terminal and internal Galβ1-4GlcNAc (LacNAc). The binding profiles of the C-terminal CRD and N-terminal CRD exhibited additional differences in binding preference. Binding assays with the C-terminal mutant DrGal9-L1 indicated that the N-terminal CRD binds to the epithelial cell surface but not to the virus. These results were confirmed using the isolated N-terminal CRD. The C-terminal CRD appears to exhibit binding preference for the epithelial cell surface but more research is necessary to confirm this, as well as determine its binding capacity for the virus. The results from a modified plaque assay on the fathead minnow epithelial cell line (EPC) revealed that while the intact recombinant Drgal9-L1 significantly increased IHNV infection of cells, a mutant Drgal9-L1 lacking a functional C-terminal CRD, or the isolated N-terminal or C-terminal CRDs neither promoted nor inhibited infection indicating that both functional CRDs are required for crosslinking to occur. Our current studies on the characterization of Drgal9-L1 CRDs binding specificity in IHNV adhesion and infection, and the identification of the signaling pathways involved in viral entry and proliferation will have wide ranging applications for aquaculture disease management and alternative vaccine developments [Supported by grant R01GM070589 from the National Institutes of Health to GRV].

### (160) Antibody-based Inhibition of Influenza H5 Hemagglutinin Binding to a Sialoglycan Receptor Using Surface Plasmon Resonance (SPR) as an Alternate to Live Virus-based Assays

Malgorzata Norton[1], Alexey Khalenkov[1], Tracy L. Kamikawa[1], Thomas Kort[2], Peter Pushko[2], Michael C. Kennedy[1] and Dorothy E. Scott[1]

*[1]Division of Plasma Protein Therapeutics, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration; [2]Medigen, Inc.*

Avian influenza continues to be a public health problem. Vaccination may prevent or ameliorate the disease; however, it may not always be feasible to match the timing of the vaccine administration to the time of exposure, or additional therapeutics may be needed in the case of severe disease. Passive antibody therapy with polyclonal anti-influenza immune (hyperimmune) globulins may be an effective complementary therapy. Hyperimmune globulin therapy is already in place for several infectious agents such as tetanus, rabies, hepatitis B, and varicella zoster. In order to screen individual plasma donations for high neutralizing titers to pool into a hyperimmune product, a fast and reliable neutralizing antibody assay is needed. Currently, the hemagluttination inhibition (HAI) and microneutralization (MN) assays are used to detect neutralizing antibodies against Influenza viruses; however, these assays require use of live virus, extensive hands-on time, and are run over multiple days. We developed a Surface Plasmon Resonance (SPR) assay to measure anti-H5 HA antibodies for neutralizing activity which has no requirement for live virus, and is rapid and automated. Briefly, biotinylated multimeric glycans containing sialic acid moieties were captured on a streptavidin-coated surface, acting as a model for influenza cell-surface receptors. Multimeric H5 HA recombinant protein micelles preincubated with a dilution sequence of antibodies or serum were then injected over the glycans. The results of the antibody dilutions preincubated with H5 HA were compared to H5 HA only, and an IC50 was calculated. Using this assay, we could measure the IC50 of anti-H5 monoclonal mouse ascites (mAb), and polyclonal rabbit, goat, ferret, chicken, and human sera. Additionally, the IC50 measurement was used to rank the mAb and polyclonal sera in order of neutralizing activity, which correlated with their rankings according to the HAI titer. In addition to its use for anti-H5 plasma screening, this SPR assay may also be of use

as a serosurveillance tool, and may be adapted to measure the neutralizing antibody against other infectious agents where the host receptor is known.

### (161) Identifying cell-surface glycans that mediate binding of Pertussis toxin

Nicole Nischan and Jennifer J. Kohler
*Department of Biochemistry, 5323 Harry Hines Blvd., Dallas, TX 75390-9185*

INTRODUCTION. Pertussis, or whooping cough, is a contagious bacterial disease without available treatment that infects about 16 million people annually worldwide with a mortality of 2% in infants younger than one year. US infections are rising to 1950s levels (41,880 cases in 2012) despite population-wide vaccination, rendering the development of treatment necessary. Reasons for increased infection rates include *B. pertussis* expressing increased levels of its key virulence factor, pertussis toxin (PT). PT is an AB5-type toxin that binds to cell surface glycans followed by cellular uptake and host cell intoxication, leading eventually to holding off the host immune response. This work seeks to identify the glycan epitope necessary for cellular binding and uptake of PT, which may allow development of a PT-neutralizing reagent.

RESULTS. To test the impact of cell-surface glycans on PT-binding, I modified the cell-surface glycans of human bronchial epithelial (HBEC) and T-lymphocyte (Jurkat) cells with inhibitors of ganglioside synthesis, the maturation of N-linked glycans, the synthesis of O-linked glycans, as well as fucosylation and sialylation. Subsequently, I assessed the impact on PT-binding by immunoblot, on-cell-ELISA or flow cytometry, and on PT-uptake by in-cell-ELISA. Inhibiting the synthesis of gangliosides or O-linked glycans had no effect on PT-binding. The inhibition of both maturation of N-linked glycans and sialylation leads to a significant reduction of PT binding and uptake; these effects are cumulative. Intriguingly, the inhibition of fucosylation leads to a 50% increase of PT binding in HBECs. In Jurkat cells, inhibition of fucosylation had no significant effect on PT-binding, however Jurkat cells have low fucose levels as shown by lectin blot. To further investigate the role of fucosylation in PT-binding, current efforts focus on stably overexpressing different fucosyltransferases in HBECs and Jurkat cells. These experiments will elucidate which fucose-epitopes protect against PT-binding.

CONCLUSIONS. N-linked glycans and sialic acid are involved in PT-binding and uptake. Fucosylation may play a protective role against PT-binding. Fucosyltransferase expression analysis will elucidate further detail about the composition of PT-binding glycans. The identification of PT's binding partners may enable the development of therapeutic agents for perturbing cell intoxication by PT.

### (162) A shotgun glycomics approach to identify influenza virus receptors in human lungs

Nan Jia[1], Lauren A. Byrd-Leotis[1], Chao Gao[1], Sandra F. Cummings[1], David A. Steinhauer[2] and Richard D. Cummings[1]
[1]*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School;* [2]*Department of Microbiology and Immunology, Emory University School of Medicine*

Influenza is a highly infectious disease that is caused by influenza viruses. Waterfowl species are the natural reservoirs of the viruses. However, at unpredictable occasions avian strains overcome species barriers and evolve the ability to transmit efficiently in mammalian species, including humans. The infection process starts with the attachment of influenza A viruses to the human airway via interactions between the viral surface protein hemagglutinin (HA) and glycan receptors that are expressed on the respiratory epithelial cells. More specifically, glycans containing terminal sialic acids have been widely accepted as the host receptors to influenza viruses. Sialic acids typically link to their adjacent monosaccharides via α2, 3 or α2, 6 glycosidic bonds and this linkage difference is thought to be a key factor contributing to the species barrier of influenza infection. In recent years, more insights have been gained to understand the initial binding step of the viruses. It is now clear that glycan sequences beyond the terminal structures also play essential roles.

A major limitation to study the initial infection process by influenza viruses is the lack of information on receptors present in the tissues that are infected in the natural host. Several attempts have been made to address this issue by extracting glycans from respiratory organs of alternative animal models or by chemically synthesising glycans. However, none of the studies have been able to define the characteristics of virus infection in natural human respiratory tissues. The goal of the present work is to exploit methods that allow for large scale preparation and purification of natural glycans to identify and characterise the receptors that are found in a human lung and determine the implications of these structural features to the binding specificity and affinity of influenza viruses.

Through this work, we have characterised a unique library of natural glycans that are expressed in human lung by mass spectrometry. To connect the structural characteristics of these glycans, we pursued a shotgun glycomics approach to identify the natural endogenous receptors to influenza viruses. Glycans were isolated from the tissue, fluorescently labelled and separated with multi-dimensional HPLC. Subsequent generation of a human lung shotgun glycan microarray allowed screening of viruses against natural glycan probes. The array data revealed differential binding of natural lung glycans to a diverse panel of influenza

viruses. Further structural analyses elucidated additional structural information of the glycan determinants that can be recognised by influenza viruses. Overall, the shotgun glycomics approach provids a powerful platform to investigate the correlation of structural characteristics of glycans with their functional aspects. [This work was supported by CEIRS grant contract HHSN272201400 to DS and RDC and NIH Grant P41GM103694 to RDC].

### (163) NanoLC-MS/MS-based Quantitative N-glycomics following 2-Aminobenzoic Acid Labeling and Methylamidation

Haiying Li, John W. Froehlich, Patricia S. Cho, Stephen A. Kostel, Shannon E. DiMartino and Richard S. Lee
*Department of Urology and The Proteomics Center, Boston Children's Hospital and Harvard Medical School*

Qualitative and/or quantitative changes in N-glycans are associated with many diseases. However, it has been a challenging task to monitor these changes due to the lack of efficient analytical techniques. We introduced a nanoLC-MS/MS-based quantitative N-glycomics strategy following an optimized dual-reaction approach. This strategy aims to achieve simultaneous quantification of neutral and sialylated glycans within a sample as well as comparative quantification of N-glycomes between two samples within one mass spectral analysis at high sensitivity and reproducibility. First, the 2-aminobenzoic acid (2-AA) "light" ($^{12}C_6$) and "heavy" ($^{13}C_6$) pair were used to differentially label two N-glycan samples. The samples were then combined and subjected to methylamidation to neutralize sialic acid residues to achieve equivalent ionization efficiency between neutral and sialylated N-glycans during MS analysis. N-glycans from a model glycoprotein, Fetuin, were used to evaluate the qualitative performance of the strategy. In total, 64 N-glycan compositions were identified in Fetuin. To test the quantitative performance of the strategy, N-glycans from a mixture of five glycoprotein standards were used, which demonstrated that the light/heavy-2-AA (alternatively) labeled glycans enabled accurate relative quantification in MS1 spectra over a 100-fold linear dynamic range. We further demonstrated the practical utility of the developed strategy by comparing the N-glycomes from a healthy human urine sample versus the same sample after removal of sialic acids by sialiase A.

### (164) GlcNDAz, a diazirine-containing sugar, can be incorporated into cell surface N-linked glycans

Han Wu[1], Amberlyn M. Wands[1], Asif Shajahan[2], Roberto Sonon[2], Parastoo Azadi[2] and Jennifer J. Kohler[1]
[1]*Department of Biochemistry, UT Southwestern Medical Center, Dallas, TX;* [2]*Complex Carbohydrate Research Center, University of Georgia, Athens, GA*

Cell surface glycans have multiple functions in various cellular processes like cell-cell interaction, cell-matrix interaction, signaling transduction, etc. N-acetylglucosamine (GlcNAc) is a common element in cell surface glycans. Previous study shows that GlcNDAz, an analog of GlcNAc functionalized by a diazirine group, can be metabolized by mammalian cells expressing mutated UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) to generate O-GlcNDAz modified proteins and crosslink these proteins to their binding partners. Results of glycopeptide analysis and crosslinking experiment indicate that GlcNDAz can also be incorporated into cell surface N-linked glycans. By crosslinking cholera toxin subunit B (CTB) and its possible receptor on T84 cell surface using GlcNDAz followed by proteomic analysis, several proteins were identified, among which crosslinking of CEACAM5 and CTB was also supported by immunoblot. This raises the possibility of using GlcNDAz to study interactions between cell surface receptors with N-linked glycan modification and their ligands.

### (165) Analysis of HIV-1 gp120 N-glycosylation in the Context of Structure and Function

Audra A. Hargett[1], Qing Wei[1], Barbora Knoppova[2], Milan Raska[1,2], Stacy Hall[1], Zina Moldoveanu[1], Zhi-Qiang Huang[1], Zhi-Qiang Huang[1], Jan Novak[1] and Matthew Renfrow[1]
[1]*University of Alabama at Birmingham;* [2]*Palacky University in Olomouc, Olomouc Czech Republic*

HIV-1 envelope (Env) is a trimeric herterodimer composed of gp41, the transmembrane protein, and gp120, the outer envelope domain. Both gp120 and the surface exposed portion of gp41 are covered in oligosaccharides which encompass 50% of the mass of gp120. These *N*-glycosylation sites (NGS) play a key role in virus entry into host cells and as a shield against broadly neutralizing antibodies (bnAbs).This "glycan shield" that is formed by the inter-linking network of *N*-glycans serve as the primary interface between the virus and host, yet the glycosylation patterns that form the shield are not well understood. We have previously demonstrated that expression of Env gp120 in a variety of cell lines result in differential *N*-glycosylation of the many potential sites within the protein and that the differential glycosylation affect Env recognition by antibodies from the sera of HIV-1-infected subjects [1] To characterize the extent of glycosylation at each NGS of the gp120 variants, we applied our high resolution mass spectrometry workflow that makes use of MS and MS/MS (CID & ETD fragmentation methods) to identify *N*-glycopeptides. By determining the percent relative abundance of each glycoform for each NGS, we are able to see glycosylation patterns based on structural location and observe changes in the glycosylation pattern when the system is perturbed by a differences in the local *N*-glycan sequon pattern. By use of this method, we were able to characterize and parameterize a combination of sequons within the high mannose patch that are interdependent and play a crucial role in the assembly of a functional trimer. These variants were analyzed using infectivity and neutralization assays. Structural modeling was used to determine the

glycan interface with the most abundant glycoforms observed by MS at these sites. This work will report on the process of interpreting a dense amount of *N*-glycan heterogeneity data and how the heterogeneity changes under different condition in the context of the protein's structure and activity.

**Reference**

[1] Raska, et al. 2010. J Biol Chem., 285:20860–20869.

### (166) Expression of the Tn Tumor Antigen in Carcinomas Is Caused by Mislocalization of Cosmc/T-synthase due to Dysregulation of MAPK Pathway

Yuliang Jiang[1,3], Sheet Kotian[3], Connie Arthur[2], Sean Stowell[2], David F. Smith[1], Guangyu An[3] and Tongzhong Ju[4,1,3]

*[1]Department of Biochemistry, Emory University School of Medicine Atlanta, GA; [2]Department of Pathology, Emory University School of Medicine Atlanta, GA; [3]Department of Oncology, the Capital Medical University Beijing Chaoyang Hospital, Beijing, China; [4]U.S. Food & Drug Administration, Silver Spring, MD*

Epithelial carcinomas are the most predominant solid tumor occurring worldwide, and exhibit a high incidence of *KRAS* mutations, mutations/over-expression of the epidermal growth factor receptors (EGFR) which result in a constitutive activation of the MAPK pathway, a hallmark of carcinomas. Interestingly, carcinomas were reported to widely express tumor-associated carbohydrate antigens, Tn and its sialylated version, SialylTn (STn). Furthermore, Tn/STn expression is associated with the progression and metastasis of the tumor cells. Yet, the molecular mechanisms underlying their expression in carcinomas are not well-understood. Here we report that Tn/STn expression is caused by the dysregulated activation of MAPKs in combination with downregulation of the MAPK negative regulator SEF, or IL17RD (interleukin-17 receptor D). IL17RD acts a scaffold to sequester MEK1/2 and ERK1/2 in normal cells, thereby regulating MAPK signaling; however, it's expression is suppressed in cancer cells, and MAPK signaling is then constitutively activated via unbound phosphorylated-ERK1/2. A rat colorectal carcinoma (CRC) cell line, LMCR+, with a *KRAS* G12D mutation, expressed STn, but had normal *Cosmc* and *T-synthase* and robust T-synthase activity. The STn expression of LMCR+ cells was eliminated only by PD0325901, a highly specific inhibitor for MEK1/2 in the MAPK pathway, but not by other protein kinases' inhibitors targeting different signal transduction pathways. Interestingly, the IL17RD was absent in LMCR+ cells, but was expressed in response to PD0325901 treatment. Furthermore, PD0325901 treatment resulted in redistribution of T-synthase activity to the Golgi fraction. In addition, transfection of LMCR+ cells with an expression vector encoding human IL17RD decreased STn expression. These results suggest that the upregulation of MAPK pathway, and downregulation of it's negative regulator IL17RD are associated with STn expression in LMCR+ cells. Importantly, the lung adenocarcinoma cell line A549 and CRC cell line HCT116, which all harbor *KRAS* mutations showed Tn expression upon introduction of *IL17RD* mutations by CRISPR/Cas9, without an significant effect on Cosmc/T-synthase expression. These results demonstrate that upregulation of MAPKs and downregulation of IL17RD in tumor cells lead to the expression of Tn and/or STn through the mis-localization of Cosmc/T-synthase, which might be one of the major mechanisms by which carcinoma cells commonly show aberrant expression of the Tn/STn tumor antigens. [This work was supported by NIH Grant U01CA207821 to TJ].

### (167) The immunomodulatory activity of ArtinM contributes to the protection against the in vitro and in vivo infection with Cryptococcus gattii

Patrícia Kellen M. Oliveira-Brito[1], Raquel A. Oliveira[1], Caroline Rezende[1], Pappanaicken Kumar[2], Maria Cristina Roque-Barreira[1] and Thiago A. da Silva[1]

*[1]Department of Cellular and Molecular Biology, School of Medicine of Ribeirão Preto, University of São Paulo – USP, Ribeirão Preto, Brazil.; [2]Department of Pediatrics-Research, MD Anderson Cancer Center, University of Texas, Houston, USA*

ArtinM from *Artocarpus heterophyllus*, a lectin that binds to the manotriose core of N-glycans, modulates immunity toward Th1 axis and confers resistance to intracellular pathogens. These properties were associated with the ArtinM binding to TLR2- and CD14-*N*-glycans on immune cells, with the consequent production of IL-12 and M1 polarization. Moreover, we known that the ArtinM effects on CD4 + and CD8 + T cells contribute for the immunomodulatory activity induced by lectin. Considering that the ArtinM activity favors the host immune response against *Paracoccidioides brasiliensis*, *Candida albicans*, and *Neospora caninum* infection, we investigated the effect of ArtinM to combat the *in vitro* and *in vivo* infection with *Cryptococcus gattii*. Initially, we evaluated the macrophages repolarization from anti-inflammatory M2 phenotype to pro- inflammatory M1 in the presence of ArtinM. We stimulated murine peritoneal macrophages with IFN-g (50 ng/mL) or IL-4 plus IL-10 (50 ng/mL) to develop M1 and M2 macrophages, respectively, and medium alone was used as a negative control. After 24 h, the cells that had previously been stimulated with IL-4 plus IL-10 were now re-stimulated with ArtinM for 48 h, and the total RNA was extracted of macrophages to quantify the relative quantification of transcripts of M1 (iNOs) and M2 (Arginase-1 and Ym-1) polarization markers. We found that the relative expression of Arginase-1 and Ym-1 mRNA induced by IL-4 plus IL-10 decreased significantly under restimulation with ArtinM whereas iNOs mRNA increased under the lectin stimulus, then ArtinM induces switching from M2 to M1 macrophages. Afterwards, we examined whether ArtinM could favor killing of *C. gattii*, and macrophages previously treated for 24 h with IFN-γ (50 ng/mL), ArtinM (2.5 μg/mL),

or the medium were infected with *C. gattii*. After 48 h of incubation, the CFU recovery from the macrophage lysate showed that the stimulation with IFN-γ or ArtinM was associated with a reduction of the CFU count compared to the unstimulated macrophages. Regarding that ArtinM-stimulated macrophages exhibit a high fungicidal activity, we evaluate whether the prophylactic administration of ArtinM can modify the course of experimental cryptococcis infection. For this, BALB/c mice received two doses of ArtinM (subcutaneous) with 7 days of intervals between each injection, and 3 days after last injection the mice were infected with *C. gattii* ($1 \times 10^3$ yeasts) via intranasal. The course of infection was evaluated every 7 days during 28 days after exposure to the *C. gattii*, and we used the pulmonary CFU recovery to measure the efficacy of the ArtinM administration. Our results demonstrated that the prophylactic administration of ArtinM reduced significantly the fungal burden in comparison to the untreated mice after 28 days of infection with *C. gattii*. The immunomodulation induced by ArtinM drives toward a Th1 response that can favor the combat against *C. gattii* infection. Support: FAPESP, CNPq, CAPES.

### (168) Using Glycan Microarrays and Molecular Dynamics to Understand the Carbohydrate Specificities of the Human Intelectins

Jonathan Viola[1], Jin Kyu Lee[2], Ryan McBride[5], Richard Cummings[4], Jamie Heimburg-Molinaro[4], James Paulson[5], Kelley Moremen[1], Amika Sood[2], Robert Woods[1] and Michael Pierce[1]

*[1]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA; [2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA; [3]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA; [4]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; [5]Department of Cell and Molecular Biology, Chemical Physiology and Immunology and Microbial Sciences, The Scripps Research Institute, La Jolla, CA*

The human immune system is comprised of two components: the innate immune system and the adaptive immune system. Upon pathogen challenge, the innate immune system component is the first line of defense. This response is nonspecific and does not create an immunological memory to the pathogen. If the pathogen successively evades the innate immune system, the human adaptive immune system is next to respond. In this case, the response is specific to the invading pathogen and creates an immunological memory. Elucidating the complexities of these essential components of the immune system may provide information for developing novel therapies to combat pathogens. There are many different lectin families showing a wide range of carbohydrate-binding specificities, functions, and tissue expression. The X-type class of lectins was first identified from *Xenopus laevis* embryos and oocytes. This *X. laevis* cortical granulelectin (XCGL-1 or XL-35) is secreted upon fertilization of the oocyte where it crosslinks the jelly coat protein thereby preventing polyspermy. To date, a total of 8 different X-type lectins in *X. laevis* have been identified with functions suggesting an involvement in the innate immune system. Interestingly, the X-type class of lectins require calcium to engage in carbohydrate binding, but do not contain a C-type carbohydrate recognition domain. There are two human homologs of XCGL-1, human intelectin-1 (hIntL-1) and human intelectin-2 (hIntL-2). hIntL-1 is a secreted and soluble lectin that is upregulated in response to pathogen infection or in response to the cytokine IL-13. We have recombinantly expressed and purified both hIntL-1 and hIntL-2 using suspension culture HEK293 cells and galactose-agarose affinity chromatography. The purified lectins were labeled with AlexaFluor-488 and analyzed on the Microbial Glycan Microarray (MGM) from the Consortium for Functional Glycomics (CFG). hIntL-1 and hIntL-2 showed binding to both Gram-negative and Gram-positive microbial glycans. Interestingly, whereas hIntL-1 showed binding to glycans containing glycerol phosphate and β-galactofuranose (β-Gal*f*) moieties, hIntL-2 did not show binding to β-Gal*f* containing glycans. Using co-crystal data of hIntL-1 and β-Gal*f* that was previously reported, we used molecular dynamics (MD) simulations to confirm a stable interaction between hIntL-1 and β-Gal*f*. We then generated a homology model of hIntL-2 from the hIntL-1 structure, and using MD simulations, we confirmed a loss of β-Gal*f* bindingdue to key amino acid substitutions in hIntL-2. We predict that both of the human intelectins are involved in pathogen surveillance, but with a biological function that remains to be determined.

### (169) Urinary Glycan Microheterogeneity and Host Susceptibility to Urinary Tract Infections

John W. Froehlich and Richard S. Lee

*Boston Children's Hospital*

High-mannose glycans in urothelium are known to be the binding epitopes for the uropathogenic e.coli bacteria that cause >90% of all urinary tract infections (UTI). Little is known about the soluble high-mannose bearing urinary glycoproteins that may competitively inhibit UPEC-urothelium interactions, however.

As a pilot study, we have analyzed urine from 14 individuals to establish a urinary glycopeptide library. These include 6 females and 8 males. These data were acquired using the Sequential Tandem Analysis of Glycopeptides (STAG) MS approach which was developed in this project. Analysis of 14,854 intact glycopeptide spectra and thousands of stub glycopeptide spectra were identified. These spectra spanned 142 glycosylation sites on 93 glycoproteins, and resulted in a total of 1,377 unique glycopeptides. The average coverage of 9.7 glycans per glycosylation site is, to the best of our knowledge, the largest average coverage per glycosylation site for any published glycoproteomics report to date, and reflects the sensitivity of the STAG approach for glycoproteomics.

Using the data obtained above, we have identified a total of 50 sites bearing high mannose glycans among 40 unique glycoproteins. These include glycoproteins from distinct tissues of origin, including circulating glycoproteins alpha-1 antitrypsin, Kininogen-1, zinc-alpha-2 glycoprotein, kallikrein and fibrinogen; urogenital glycoproteins including uromodulin and prostatic acid phosphatase; and plasma membrane glycoproteins which are likely produced in urothelial tissues. One critical outcome of this data is that urine is clearly useful for probing glycoproteins which are synthesized in diverse tissues. Based on these data, we have observed glycoproteins which were synthesized in the liver, B cells, T cells, the kidney, the urothelium, and the prostate, all of which harbor some level of the requisite high mannose type glycans needed for UTI protection. This suggests that alterations in diverse tissues has the potential to affect UTI protection.

A total of 17 healthy controls and 17 pediatric patients with recurrent UTI were subsequently selected for a detailed biomarker study. Application of the STAG approach and our informatics platform, glycoPipe were utilized for all analyses.

### (170) Glycoprofiling of Dirofilaria immitis and infected dog sera for the identification of new drug targets and glycobiomarkers

Anna-Janina Behrens[1], Max Crispin[2], Christopher Taron[1] and Jeremy M. Foster[1]

*[1]New England Biolabs; [2]University of Oxford*

Filiariases are diseases caused by infection with filarial nematodes transmitted by insect vectors. The filarial roundworm *Dirofilaria immitis* causes heartworm disease in dogs and other carnivore animals that likely end fatal. *D. immitis* is closely related to *Brugia malayi*, a causative agent of lymphatic filariasis that afflicts more than 40 million humans. Little is currently known about the glycosylation of these filarial nematodes, although their glycans are likely important for evading host immune surveillance as well as in host-parasite interactions. Detailed information on the composition and structure of filarial glycans can lead to the identification of new drug targets. Major drawbacks of currently available drugs include limitations as to which life stage of the parasite they target and adverse side effects. Our studies employ a set of different mass spectrometry approaches to reveal fine details on filarial glycosylation. Additionally, we are characterizing the glycosylation profiles of infected host sera with the aim of identifying new glycobiomarkers that can improve current diagnostic methods.

### (171) HUMAN ADENOVIRUS TYPE 5 INCREASES CELL HOST FUCOSYLATION

Gutiérrez Huante K. and Gonzalez R. A. Martínez Duncker I

*Cell Dynamics Research Center, Morelos State Autonomous University. Av. Universidad 1001, Cuernavaca, Morelos, 62209. Tel. 3297000, ext.3364*

The carbohydrate moieties of glycoconjugates modulate important cellular processes (eg. differentiation, proliferation or pathogen cell-host interactions), and their synthesis and structure is subject to genetic and environmental regulatory events. Few studies have described and addressed the importance of changes in the glycosylation profile of virus infected cells. Infection with CMV, VZV and other herpes viruses, as well as HCV has been reported to increase the expression of fucosylated epitopes, like $sLe^x$ (Siaa2-3Galb1-4(Fuca1-3)GlcNAcb1-R) and $Le^y$ (Fuca1-2Galb1-4(Fuca1-3) GlcNAcb1-R), through the regulation of specific host cell fucosyltranferases (FUTs). The increased expression of these epitopes has been proposed to favor the dissemination of viral progeny although more detailed studies are required to unravel the biological roles of these changes.

In this work, we assess how host cell glycosylation is affected by infection with Human adenovirus type 5 (HAdV-C5). HAdV-C5 infection is the most commonly studied vector for gene therapy, vaccines and anti-cancer vector development, as well as an outstanding model for the study of gene expression and structure. Infection with this virus notably causes respiratory diseases as well as epidemic keratoconjunctivitis (EKC) and gastroenteritis. The infection is relatively mild and limiting in immunocompetent infants, children and adults, whereas in patients who are immunocompromised infection may be severe or even fatal. We demonstrate for the first time that HAdV-C5 infection modifies the glycosylation profile of A549 cells, used as a model of human lung epithelium. Using carbohydrate metabolic labeling and a lectin panel (*AAL*, *UEA-I* and *LCA*) we determined that HAdV-C5 infection causes a significant increase in cell surface fucose and that this increase would be at the expense of Fucose in a1-2 linkage as demonstrated with UEA-I lectin staining. This data led us to assess the expression of the well-known bi-fucosylated $Le^y$ antigen, associated with differentiation, migration, adhesion and pathological processes such as cancer. Through inactivation experiments we demonstrate that these glycosylation changes are dependent on the expression of viral genes. We also report the genetic basis of the observed fucosylation changes and the potential glycoprotein candidates affected by them. This work opens several questions regarding how increased fucosylation participates in the pathogenesis caused by adenovirus infection and its implications in biotechnology and medical therapy.

### (172) Correlations between the MGAT3 and BACH2 promoter methylation and IgG glycans suggest the role of these genes in IgG glycosylation and inflammatory bowel disease

Vlatka Zoldoš[1], Dora Markulin[1], Marija Klasić[1], Aleksandar Vojta[1], Irena Trbojević-Akmačić[2], N. Ventham[3], N. Kennedy[3], J. Satsangi[3], V. Annesse[4], S. Pinho[5] and Gordan Lauc[2]

*[1]University of Zagreb, Faculty of Science, Department of Biology, Division of Molecular Biology, Horvatovac 102 A,*

*10 000 Zagreb, Croatia; [2]Genos Ltd, Glycobiology Laboratory, Zagreb, Croatia; [3]University of Edinburgh, Faculty of Science, Edinburgh, UK; [4]University of Carregi, Florence, Italy; [5]IPATIMUM, Lisbon, Portugal*

Many genome-wide association studies (GWAS), genome-wide methylation studies, and studies of promoter methylation of candidate genes for inflammatory bowel disease (IBD) revealed genetic and epigenetic component playing role in this disease. However, the molecular mechanisms behind the IBD pathology are still insufficiently understood. The immune system, microbiome, and the environment are significantly involved in IBD, too. Interestingly, independent GWA studies identified several genes to be associated with both IBD and IgG glycosylation, including the *MGAT3* and *BACH2* genes. The *MGA*T3 is an *N*-acetilglukosaminyltransferase III (GnT-III), which adds bisecting GlcNAc on β1,4-linked mannose in three-mannose core of *N*-glycans, and this significantly change function of the glycan on a glycoprotein. The *BACH2* is a transcription factor, a master regulator of gene network important for B cell integrity and terminal differentiation to plasma cells. Using bisulfite pyrosequencing, we analyzed CpG methylation in the promoter of these two genes from peripheral blood of several hundred IBD patients and healthy controls from two large independent cohorts. For both genes, significant differences in the methylation level were found between both, Crohn's disease (CD) and ulcerative colitis (UC) comparing with healthy controls (HC). In addition, the same pattern of the methylation change was recorded for the both genes in CD + 19 B cells isolated from the whole blood of the subset of IBD patients. The correlation study was performed on the *MGAT3* and *BACH2* promoter methylation and the IgG glycan data obtained for the same individuals of the two large cohorts. The *MGAT3* promoter methylation correlated significantly with the FA2B/FA2 ratio, suggesting that activity of the GnT-III enzyme might be altered in IBD. Significant correlation was also found with galactosylated and sialylated structures, suggesting that these processes could be co-regulated with the *MGAT3* expression. The correlations between the *BACH2* promoter methylation and IgG glycans were less obvious. This is easy to explain since the *BACH2* is not a glycosyltransferase, therefore it does not have a direct effect on IgG glycosylation, but probably indirectly regulates this process through transcriptional regulation of some glyco-genes. Our results suggest that the anti-inflammatory character of IgG is decreased in IBD through decrease in galacosylation and sialylation, and an increase of bisecting GlcNAc on digalactosylated glycan structures, and this could be a result of epigenetic deregulation of the key genes. Finally, we showed that CpG methylation in the promoter of the *MGAT3* gene is altered in CD + 3 T cells isolated from inflamed bowel tissue of UC patients of the third smaller cohort, for which biopsies were available, suggesting a functional role of this glyco-gene in IBD pathogenesis.

**(173) Multivalent substrates for protein glycosylation: new avenues in substrate engineering and fundamental insight in mechanism of Actinobacillus pleuropnomoniae N-glycosyltransferase**

Hanne L.P. Tytgat, Chia-Wei Lin, Jacqueline Mock, Timothy G. Keys and Markus Aebi
*Institute of Microbiology, ETH Zurich, Zurich, Switzerland*

Multivalency is a common theme in receptor-ligand interactions in nature, with one of the prominent examples being lectins recognizing multivalent carbohydrate ligands. Here, we designed a series of multivalent tags to investigate if we can create multivalent glycosylated proteins using the cytosolic *N*-glycosyltransferase from *Actinobacillus pleuropneumoniae* (ApNGT). The tags consist of simple sequence repeats including an *N*-glycosylation sequon N-X-S/T and 0–5 spacing amino acids (e.g. $[\underline{N}AT]_5$, $[TAGA\underline{N}ATA]_5$). For analysis of glycosylation site occupancy, the tags were attached to the C-terminal of GFP.

Site occupancy was evaluated using immunoblot, UPLC and mass spectrometry, thus revealing the ability of the ApNGT to modify the tags. Interestingly, the ApNGT is able to modify all glycosites of all tags, illustrating the efficacy of the enzyme for glycoengineering purposes and of the tags for substrate engineering. Dedicated MS/MS analysis on the site occupancy of the glycoforms of the tags, generated fundamental insights on the ApNGT enzyme mechanism. These in-depth analyses suggested that the enzyme may processively modify its targets.

From an engineering perspective, the tags form an innovative new addition to the glycoengineering toolbox, having the potential to transform virtually any protein into a multivalently glycosylated protein. The tags will be furthermore explored and engineered to generate specific ligands for existing multivalent lectins, like Siglecs and Galectins.

**(174) Reproduction of an L-Rhamnose and D-Galactose-specific Lectin from a Lost Strain of Streptomyces**

Yoko Fujita-Yamaguchi[1,2], Karine Bagramyan[3], Yoshiki Yamaguchi[4], Akemi Ikeda[4], Teresa B. Hong[3] and Markus Kalkum[3]
*[1]Department of Molecular & Cellular Biology, BRI of City of Hope; [2]Department of Diabetes Complications & Metabolism, BRI of City of Hope; [3]Department of Molecular Immunology, BRI of City of Hope, Durate, CA 91010, USA; [4]Structural Glycobiology Team, Systems Glycobiology Research Group, RIKEN Global Research Cluster, RIKEN, Saitama 351-0198, Japan*

Blood type B-specific *Streptomyces* S27S5 hemagglutinin (SHA) was discovered and characterized in the 1970s. Although the strain 27S5 has since been lost, the purified SHA protein survived intact under frozen conditions and retained its activity. Last year, we reported the following. 1) FTICR MS analysis determined a molecular mass of

13,314.67 Da. 2) MS methods, *Streptomyces* genomic database analyses and N-terminal sequencing solved the primary structure of SHA consisting of 131-amino acids. SHA is homologous to N-terminally truncated hypothetical proteins encoded by the genomes of *S. lavendulae*, and others. 3) The closest homologue (a putative polysaccharide deacetylase, PDSL) in *S. lavendulae* encodes 68 additional N-terminal amino acids. 4) SHA domain of PDSL, SHA(PDSL), was expressed in *E. coli* and purified. Comparison of peptides derived from SHA and SHA(PDSL) revealed a single A-to-E amino acid difference. 5) SHA(PDSL)-A108E transiently expressed in HEK293S cells exhibited binding activity to gum arabic gels which was and is used to purify SHA, suggesting successful construction of SHA gene.

In this study, the mutant SHA(PDSL)-A108E was expressed in *E. coli* by transforming with a novel construct that encoded an *E. coli* codon optimized, His-tagged, Trx-SMT3(SUMO family protein)-fusion protein. The expressed fusion protein was purified using His6-tag specific nickel-NTA column from transformed cells after solubilization with 5 M urea/B-PER lysis buffer, followed by refolding on the nickel-NTA column in presence of D-galactose. Soluble recombinant SHA (rSHA) was cleaved off from the Trx-SMT-3 portion by digestion with His-tagged Ulp1. The purified rSHA was analyzed together with the authentic SHA for their purity and binding specificity using RayBio Glycan Array 100. The microarray results clearly revealed that rSHA has blood-type B carbohydrate-specificities and high affinity to L-rhamnose comparable to SHA. Therefore, rSHA represents the native SHA produced by the lost *S.* sp. 27S5 strain.

Furthermore, we prepared a GFP-SHA fusion protein to show its binding to microbial surfaces, indicating that rSHA provides a valuable resource for developing novel diagnostics and therapeutics for galactomannan and L-rhamnose-containing microorganisms.

The primary sequence of SHA is made of three homologous "SHA domains", each consisting of 29 to 33 amino acids. Together, they comprise 92 amino acids, 70% of the SHA molecule. SHA domains show homology to tryptophan-rich ChW domains. Tryptophan-rich nature of SHA and the role of tryptophans in L-rhamonose binding to SHA were reported four decades ago. To date no conclusive study has been published as to the role of ChW domains, although carbohydrate recognition functions have been suggested. Therfore, our rSHA will provide a unique opportunity for studying the role of ChW and SHA domains in carbohydrate binding.

### (175) Comprehensive Analysis for Structural Characterization of Peptidoglycan

Sara Porfirio, Stephanie Archer-Hartmann, Russell W. Carlson and Parastoo Azadi
*Complex Carbohydrate Research Center, UGA*

Peptidoglycan (PG) is a structural polysaccharide found in the cell wall of both Gram-positive and Gram-negative bacteria. It is formed by glycan strands of repeating units of the disaccharide GlcNAc-MurNAc, which are connected by peptide bridges with a varying degree of cross-linking. This complex structure is further modified through chemical variation of the glycan chains. Possible modifications include deacetylation, glycolylation and attachment of surface polymers, such as teichoic acids.

Peptidoglycan is important in preserving the integrity of bacterial cells by withstanding turgor pressure, and any change that affects PG biosynthesis or degradation during cell growth will disturb cell viability. Because of its function, PG is also the target of many antimicrobial drugs and it is therefore imperative to understand the fundamental structure of this polysaccharide. To this day, the number of species whose PG fine structure is fully characterized is still limited and there are also few comparative studies which survey different bacterial species. Considering the major role of PG in bacterial cell structure and integrity, the study of this molecule is of utmost relevance, with prospective ramifications to several disciplines such as taxonomy, physiology, pharmacology and pathogenesis.

In the past decades, the development of analytical techniques such as cryo-TEM, HPLC and MS has allowed to explore previously unknown features of PG structure as well as to better characterize the building blocks of this fascinating molecule. However, the process of muropeptide isolation by HPLC and further MS analysis is lengthy and tedious. Most recently, automated techniques such as LC-MS and data processing softwares (Byonic) have been successfully applied to PG analysis, thus significantly improving its speed.

Here we present a strategy for comprehensive analysis of PG, aiming at characterizing PG structure using different techniques including GC-MS and high-resolution mass spectrometry (LC-MS, MALDI-TOF-MS, NSI-MS-MS). Amino acid and glycosyl composition analysis of PG samples by GC-MS analysis of heptafluorobutyrate derivatives can be used to confirm sample purity and quantify ratios of PG components. Further structural characterization is accomplished using MALDI-TOF-MS and LC-MS analysis to determine the structure of isolated muropeptides and predict possible modifications at the peptide or glycan level, using data analysis softwares to facilitate data interpretation.

### (176) Determining the structure of Cryptococcus neoformans polysaccharide capsule

Maggie P. Wear[1], Radames JB Cordero[1], Anthony Bowen[3], Anne Jedlicka[4], Marcos D. Battistel[2], Aaron Marcella[2], Lorenzo Guazzelli[5], Stefan Oscarson[5], Darón I. Freedberg[2] and Artruo Casadevall[1]
[1]*Molecular Microbiology and Immunology Department, Johns Hopkins Bloomberg School of Public Health;* [2]*Center for Biologics Evaluation and Research, U.S. Food and Drug Administration;* [3]*Department of Microbiology and Immunology, Albert Einstein College of Medicine;* [4]*Genomic Analysis and Sequencing Core Facility, Johns*

*Hopkins Bloomberg School of Public Health; [5]Centre for Synthesis and Chemical Biology, University College Dublin*

The pathogenic fungus *Cryptococcus neoformans* causes disseminated cryptococcal meningitis in immunocompromised indivduals. The polysaccharide capsule, mostly composed of glucoxylomannan (GXM), is a critical virulence factor in *C. neoformans* infections. Seminal work by Robert Cherniak and colleagues in the 1980s and 90 s identified seven oligosaccharide repeat units for GXM, called motifs. While we have known these GXM motifs for nearly twenty years, and our lab had developed many antibodies targeting the capsule, the macromolecular structure of GXM remains unknown. Here we utilize two approaches to examine GXM; first a synthetic glycan array building on the known GXM motifs, and second, fractionation and analysis of GXM subunits taken directly from *C. neoformans* exopolysaccharide. Using the glycan array and assessing antibody binding to printed synthetic glycans, we have identified new extended GXM motifs. One of these synthetic oligosaccharides was then examined by NMR and it's three-dimensional structure solved. To our knowledge, this is the longest branched oligosaccharide for which the three-dimmensional structure has been determined. HPLC fractionation and mass spectrometry analysis of purified exopolysaccharide fractions varying from less than 1 kDa to 10 kDa in size shows both smaller motifs than previously reported, and long polysaccharide chains containing many motifs. Utilizing these complementary approaches, a new macromolecular structure of *C. neoformans* capsular GXM is emerging.

### (177) Intact and Native Mass Analysis of Glycoproteins

Marshall Bern[1], Yong J. Kil[1], Tomislav Caval[2], Vojtech Franc[2] and Albert J.R. Heck[2]
*[1]Protein Metrics Inc.; [2]Utrecht University*

Intact mass analysis provides a simultaneous and quantitative view of a protein's major proteoforms, including variations in the total mass of glycosylation. Intact mass spectra vary widely with solution conditions. Denaturing conditions give fewer adducts, higher charge states, and stronger overall signal. Native conditions preserve noncovalent binding and original folded structure of the proteins, which results in a much narrower charge state distribution and a lower charge state. Lower charge state provides more space between adjacent charge states, allowing separation of protein ions coming from a heterogeneous mass distribution. Regardless of native or denaturing conditions, resolution and sensitivity depend upon the mass spectrometry instrument and the charge deconvolution algorithm. Here we show the effectiveness of native MS for the analysis of complex glycoproteins, including mAbs and other biotherapeutics. We analyze dimerization, glycosylation site occupancy, and amount and heterogeneity of sialylation, without the biases due to differing ionization propensities inherent in peptide-level approaches. We also show the advantages of a new "parsimonious" charge deconvolution algorithm, which gives fewer artifacts and more accurate peak shapes and intensities than the standard MaxEnt algorithm that has been in widespread use for the past 25 years.

### (178) Are polysialylated exosomes an endogenous defense mechanism against neutrophil extracellular traps?

Kristina Zlatina[1,2], Max Saftenberger[2], Christina E. Galuska[1,2], Jan Dambon[1,2], Andrea Kühnle[1], Thomas Lütteke[3] and Sebastian P. Galuska[1,2]
*[1]Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology; [2]Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-University; [3]Institute of Veterinary Physiology and Biochemistry, Justus-Liebig-University*

One mechanism of the innate immune system during the fight against pathogens is the formation of neutrophil extracellular traps (NET). This meshwork of chromatin, together with antimicrobial peptides and enzymes, efficiently traps protozoa, fungi and bacteria and facilitates their elimination. However, it was observed that an excessive NET formation can induce cell damage, leading to inflammatory disorders. Therefore, in search of an approach to reduce the adverse outcome of NET, for example external antibodies against histones, enzyme inhibitors as well as polysialic acid (polySia) were discussed. But does the organism also have possibilities to counteract the cytotoxicity of NET by itself?

Interestingly, our examinations on exosomes isolated from blood samples indicated that these vesicles can be carriers of polySia and bind to NET in a polySia dependent mechanism. The polySia mediated accumulation on NET was confirmed using polysialylated fluorescent beads. Moreover, we observed that polysialylated exosomes are capable of reducing histone-mediated cytotoxicity. These results may be useful e.g. to enrich pharmacological substances on NET via polysialylated particles, aiming to influence excessive inflammatory disorders.

### (179) Site-specific detection of advanced glycation endproducts with newly developed single-chain variable fragment antibodies

Ulrika Wendel[1], Nina Persson[1], Christian Risinger[1], Ekaterina Mirgorodskaya[2], Carina Sihlbom[2], Eva Bengtsson[3], Björn Nodin[4], Lena Danielsson[5], Charlotte Welinder[4,6], Maria Panico[7], Anne Dell[7], Stuart Haslam[7], Gunilla Nordin Fredrikson[3], Bo Jansson[4] and Ola Blixt[1]
*[1]Chemical Glyco-Biology Laboratory, Department of Chemistry, Faculty of Science, Copenhagen University, Denmark; [2]The Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg, Sweden; [3]Department of Clinical Sciences, Scania University Hospital, Malmö Lund University, Sweden; [4]Division of Oncology and Pathology, Dept. of Clinical Sciences, Lund University, Sweden; [5]Division of Clinical Chemistry and Pharmacology, Dept. of Laboratory Medicine, Lund University, Sweden; [6]Centre of Excellence in Biological and Medical Mass*

*Spectrometry "CEBMMS", Biomedical Centre D13, Lund University, Sweden; [7]Department of Life Sciences, Imperial College London, South Kensington Campus, UK*

Advanced glycation endproducts (AGE) are formed by non-enzymatic reactions between carbohydrates and protein amines, causing changes in protein structure and charge, antibody responses, and inflammation by binding to receptors. AGEs are associated with progression and complications in diseases such as diabetes, atherosclerosis, cancer, Alzheimer's disease, and thus have potential as clinical markers. Current methods for glycation detection are limited since they often measure (i) total glycation instead of specific glycated sites and (ii) early glycations and not the later advancedglycations. Monoclonal antibodies are useful for site-specific detection, however the existing anti-AGE antibodies are poorly characterized regarding binding patterns.

The aim of this study is to develop monoclonal single-chain variable fragment antibodies (scFv:s) that recognize specific AGE sites, using a phage-display platform, microarray technology and highly characterized target libraries. In this way, novel antibody candidates will be produced for improved diagnostics, therapeutics and understanding of AGE-related diseases.

Phage libraries were produced from mice immunized with AGE-modified proteins. Peptide libraries were synthesized by solid phase peptide synthesis using glycated amino acid building blocks. Proteins were glycated *in vitro* with carbohydrates and characterized by SDS-PAGE and LC-MS/MS. Phage libraries were selected for AGE-binding clones using a high-throughput phage-display platform with concurrent microarray evaluation. After conversion into soluble scFv:s, interesting clones were expressed and further screened and characterized for their binding patterns against comprehensive glycated peptide and protein libraries. Final candidates were evaluated with immunohistochemistry of relevant biological material.

The immunized mice developed immune responses against AGE-structures, whereby an antibody phage display library could be constructed from their spleens. Modified lysine and arginine residues from low- and high-concentration protein glycations, were identified with LC-MS/MS. Modifications of highly glycated protein samples were recognized with SDS-PAGE. Microarray evaluation of the phage pools from the selection showed binding to AGE structures on peptides, blood proteins and histones. Screening and characterization of selected scFv:s, using comprehensive AGE-target microarrays, revealed several antibodies with specificities for AGE structures. One of the clones, with a preference for a site containing a CML or a CEL modification, showed clear staining of mouse atherosclerotic plaques and of a tissue microarray from 82 human pancreatic tumors.

We have identified promising antibody candidates, recognizing AGE modifications at specific sites. Binding patterns were identified by microarray analysis against glycated peptides and proteins, characterized by LC-MS/MS and SDS-PAGE.

### (180) New insect cell line to produce recombinant glycoproteins with EndoH sensitive N-glycans

Hideaki Mabashi-Asazuma[1] and Donald L. Jarvis[2,3]
*[1]Ochanomizu University, Tokyo, Japan; [2]University of Wyoming, Laramie, WY; [3]GlycoBac, LLC, Laramie, WY*

The heterogeneous and mobile nature of glycans hampers their structural analysis using crystallography. The main solution to these problems has been to produce recombinant glycoproteins using mammalian cells with an MGAT1 mutation, such as the Lec1 cell line. Another solution has been to use insect cells, which add relatively uniform and small glycans. In this study, we enabled a combinatorial approach by using CRISPR-Cas to isolate SfLec1 cells, which are insect cells with a mutant SfMGAT1 gene. SfLec1 cells produce only high mannose type chain-glycans, mainly Man5, and no Man3, which is the structure normally produced by insect cells. When SfLec1 cells were used as a host in the baculovirus insect cell system (BICS), recombinant human erythropoietin acquired mainly Man 5 and all its chain-glycans could be removed with EndoH. Similar results were obtained with human IgG1-Fc and mouse IgG2a-Fc. SfLec1 expands the utility of BICS by enabling us to selectively produce glycoproteins with two different glycoforms and is a promising expression system for structural analysis of glycoproteins. Moreover, SfLec1 products can be used as substrates for glycan remodeling using ENGase mutants.

### (181) Deep sequencing of proteoglycans

Joshua A. Klein, Le Meng and Joseph Zaia
*Boston University*

Proteoglycans play critical multifaceted roles in maintaining extracellular matrix and regulating cellular functions including growth, signaling, and tissue patterning. Dysregulation of proteoglycan structure is key to inflammation, cancer, and neurodegeneration. However, the peptide sequence coverage of complex proteoglycans, including aggrecan and versican, are poorly revealed by mass spectrometry-based proteomics studies. The major hurdle is the high degree of modification by glycosaminoglycans (GAGs). For example, aggrecan has more than 100 GAG chains and several sites of *N*- and mucin-type *O*-glycosylation on its core protein, of which only a small portion of this protein has been identified in database such as Swiss-Prot and PeptideAtlas.

We developed a novel workflow to improve sequence coverage and identification of GAG sequons in proteoglycans. Bovine aggrecan or enriched proteoglycans from bovine cortex lysates were digested with a combination of trypsin (or chymotrypsin) and Glu-C according to manufacturers' instructions. Peptide products were separated with a 10 kDa cut-off spin filter tube and the top portion was further digested with heparin lyases and chondroitinase ABC, which left a tetrasaccharide linker on these peptides. Peptides were cleaned with C-18 ziptip and analyzed with Thermo nLC easy 1200 and Q Exactive HF. Raw data were analyzed using PEAKS and our house-made

GlycReSoft with customized modifications for GAG linker peptides. The FDR was set at 0.1% at peptide level.

Aggrecan is a member of the hyalectan family of large aggregating proteoglycans. Found in most connective tissues as well as brain, aggrecan contains three globular domains and an extended GAG attachment region. Most of the information on the glycosylation structure of aggrecan comes from classic studies on cartilage. There is little information regarding tissue and disease-specific glycosylation patterns of aggrecan and other hyalectans. Public proteomics knowledge-bases contain aggrecan peptides present in the globular domains (G1, G2 and G3) but lack peptide coverage in the GAG attachment CS-1 and CS-2 domains. Using our optimized workflow, we detected more than 150 peptides in the CS-1 and CS-2 domains of aggrecan that have never been reported in Swiss-Prot and PeptideAtlas. Most importantly, we detected more than 100 peptide sequences that bear CS chains, which have not been reported before. Interestingly, these peptides showed high micro-heterogeneity in their GAG tetrasaccharide linkers. Next, we investigated deep sequencing of proteoglycans in complex whole tissue lysate. Proteoglycans enrichment significantly improved peptide coverage in proteoglycans and identification of GAG sequons. These results illustrate the ability to define the molecular state of complex proteoglycans in a given context.

### (182) Harnessing the Power of Natural Selection to Define and Optimize Sialoglycan-Recognizing Probes (SGRPs) for Exploring the Biology, Physiology and Pathology of the Dynamic Sialoglycome

Saurabh Srivastava[1], Andrea Verhagen[1], Brian Wasik[2], Hai Yu[3], Aniruddha Sasmal[1], Barbara Bensing[4], Naazneen Khan[1], Zahra Khedri[1], Sandra Diaz[1], Paul Sullam[4], Nissi Varki[1], Xi Chen[3], Colin Parrish[2] and Ajit Varki[1]

*[1]Glycobiology Research and Training Center, University of California, San Diego, San Diego, CA; [2]College of Veterinary Medicine, Cornell University, Ithaca, NY; [3]Department of Chemistry, University of California-Davis, Davis, CA; [4]School of medicine, University of California, San Francisco, San Francisco, CA*

Cell surface or secreted glycans contribute significantly to diverse biological processes, yet remain poorly explored, partly due to structural complexities and technical limitations. A prominent example is the diverse array of terminal sialic acids (Sias), and their various linkages to underlying glycans (sialoglycans), which remain largely intractable to full analysis. Most state-of-the-art glycomic methods are powerful but require specialized expertise, can destroy or miss many aspects of Sia diversity, and do not elucidate the intact sialoglycome in a native state. Glycan-recognizing protein probes are easy to use, even for non-specialists. However, traditional sialoglycan-recognizing probes (SGRPs) are plant lectins such as MAL and SNA, which only define a limited range of Sia complexity and whose natural ligands are unknown; or, antibodies with restricted specificity. Lacking a simple and systematic approach towards in situ identification, tracking and analysis of sialoglycans, many researchers tend to avoid these molecules in their studies. We aimed to select, collect and optimize a well-defined set of recombinant and stably tagged SGRPs with specific requirements for Sia modifications or linkages in their binding epitope, which can be used as a practical toolkit by non-experts. Reasoning that strong natural selection and Red Queeneffects during evolution would have already maximized their specificity and avidity, we focused particularly on microbial proteins: bacterial adhesins and toxins, viral hemagglutinins, viral hemagglutinin-esterases and phage proteins, resorting to vertebrate proteins only when necessary. SGRP specificity is also confirmed by a sialoglycan array displaying many aspects of the natural diversity (>120 sialoglycans). An emphasis is on experimental conditions that ensure minimal loss or damage of relatively unstable Sias such as O-acetylated ones. We have so far defined 10 probe classes (SGRP1-SGRP10) identifying Sias with various underlying linkages and modifications. The SGRPs are being characterized by general methods such as ELISA, Western Blot and FACS using cell lines, sera and RBCs of various species. Probes are screened in parallel to a nonbinding mutant of the same protein, and compared with commonly used plant lectins. The data so far not only demonstrate high order specificity of SGRPs towards samples from different sources but also indicate the unpredictable variance in sialoglycans among closely related samples. Results emphasize that these SGRPs could discriminate among sialoglycans differing minutely in their underlying structures. SGRPs are also being analyzed by immunohistochemistry of mouse tissues including those with genetically-induced changes. This work will provide a simple and reliable toolkit to track the sialoglycome in biological samples. Our larger aim is to demonstrate the efficacy of the toolkit to study dynamics of the sialoglycome in different physiological and pathological states.

### (183) DrawGlycan-SNFG: Aiding mass spectrometry data analysis by rendering glycans and glycopeptides with fragmentation information

Kai Cheng, Yusen Zhou and Sriram Neelamegham

*Department of Chemical and Biological Engineering, Clinical and Translational Research Center, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA*

Glycan or carbohydrate structures can be pictorially represented using symbolic nomenclatures. The symbol nomenclature for glycans (SNFG) contains ~75 different monosaccharides represented using various colors and geometric shapes. A simple tool to convert International Union of Pure and Applied Chemistry (IUPAC) format text to SNFG will be useful for sketching glycans and glycopeptides. Such code can also enable the development of more sophisticated applications,

where the visual representation of carbohydrate structures is necessary. Such applications include tools for pathway visualization and mass spectrometry (MS) data analysis. To address these needs, the current presentation describes DrawGlycan-SNFG, a freely available, platform-independent, open-source tool. It allows the display of glycans and glycopeptides from IUPAC-condensed text inputs for straight-forward, clean, and rapid sketching of a variety of carbohydrate structures. This includes branched, repeating and ambiguous glycan structure assignments. The software also allows the rendering of glycan and glycopeptide fragments. Examples will be presented for how this can be integrated into MS based data analysis programs for the automated annotation of MS spectrum related to glycans and glycopeptides. Overall, DrawGlycan-SNFG will be useful for experimentalists looking for a ready to use, simple program for sketching carbohydrates. It will also be useful for software developers interested in incorporating SNFG into their program suite. Availability: The online version of this program is provided with a user-friendly web interface at www.virtualglycome.org/DrawGlycan. Downloadable, standalone GUI (Graphical User Interface) version and the program source code are also available from this repository.

### (184) GLiCo-Click and GlycoFRET - Glycan-Mediated Ligand-Directed Target Identification and Target Engagement Assays

Henning Stockmann, Violeta Marin, Viktor Todorovic, Victoria Scott, Clare Gerstein, Marc Lake, Leyu Wang, Ramkrishna Sadhukhan, Paul Nimmer, Corina Balut, Paul Richardson and Anil Vasudevan
*AbbVie*

Although >40% of marketed small-molecule drugs target plasma membrane proteins, traditional target identification strategies based on ligand affinity capture and mass spectrometry are not well-suited for multi-pass membrane targets at the cell-surface. In addition, developing target engagement screening assays with suitable throughput and sensitivity in native cells can be challenging with ligands that interact non-specifically with the cells, or with targets of low abundance in non-engineered cell-systems. In order to overcome these limitations, we have used in situ metabolic labeling of cell-surface glycans to non-selectively incorporate bioorthogonal azides into cell-surface proteins. In the case of target identification experiments, a ligand of interest is modified with an affinity tag, and a strained alkyne whose reactivity is tuned to exploit the relatively high local concentration of a ligand-bound target relative to the ligand in bulk solution. Incubation of the ligand with azide modified cells results in specific crosslinking of the ligand to its protein target allowing subsequent enrichment and identification via a method called glycan-mediated ligand-controlled click GLiCo-Click chemistry.

In another manifestation of the method we used the azide-modified surface proteins to non-specifically incorporate a long-lifetime lanthanide fluorophore. By incorporating an acceptor dye into a ligand we developed highly selective time-resolved fluorescence assays (TR-FRET) for several targets with excellent sensitivity and high signal-to background ratios to provide GlycoFRET assays that could be used in non-engineered cells.

### (185) Glycan analog libraries for the development of high affinity ligands of glycan binding proteins

Corwin M. Nycholat[1], Shiteng Duan[1], Sam Moons[1], Buyankhishig Tsogtbaatar[1], Eike Wamoff[4], Grant Bare[2], Ryan McBride[1], Kevin Worrell[1], Christoph Rademacher[4], Sean Stowell[3], Barry Sharpless[2] and James C. Paulson[1]
*[1]Department of Molecular Medicine, The Scripps Research Institute, La Jolla CA; [2]Department of Chemistry, The Scripps Research Institute, La Jolla CA; [3]Pathology Department, Emory University School of Medicine, Atlanta GA; [4]Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Potsdam GE*

Lectins, including for example, the siglecs and galectins, are a major class of glycan binding proteins (GBPs) that commonly bind to glycan epitopes present on *N*- and *O*-linked glycoproteins, and glycolipids. The carbohydrate recognition domain (CRD) or glycan binding site of these lectins interact with glycan structures to mediate a diversity of physiological and pathological processes.[1,2] Though in many cases single-site binding affinities to 'natural' glycans are low (μM-mM range), multivalent interactions between multiple CRDs and glycans can yield functional high avidity binding *in vivo*.[3,4] High affinity and selective ligands of GBPs that can block their interactions with glycans *in vitro* or *in vivo* are valuable tools for investigating their functions or serve as potential therapeutics. Potent inhibitors of various GBPs have been developed based on glycan analogs which are glycans modified with non-carbohydrate substituents that bind to sub-sites adjacent to the core-conserved CRD.[5,6]

To develop ligands of galectins and siglecs, we have chemoenzymatically synthesized a diverse library of glycan analogs using LacNAc and sialyl LacNAc scaffolds, respectively. We use a combinatorial approach to synthesize analogs based on sulfonamide, urea, thiourea, SuFEx,[7] and CuAAC chemistry. Additionally, we have conducted an *in-silico* screen of a commercial building block library guided by existing galectin co-crystal structures to identify favorable amide linked substituents. Here we will describe the synthesis of these glycan analog libraries and glycan microarray screening against recombinant galectins and siglecs. (NIH Common Fund grant HL136272 and NHLBI grant HL107151).

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### (186) Development of a New Human CD22 transgenic Mouse

Matthew S. Macauley[1,5], Kyle J. Bednar[1,2], Elena Shanina[1], Romain Ballet[4], Edward P. Connors[1], Shiteng Duan[1], Joana Juan[1], Britni M. Arlian[1], Mike D. Kulis[3], Eugene C. Butcher[4], Wai-Ping Fung-Leung[2], Tadimeti S. Rao[2] and James C. Paulson[1]

*[1]Department of Molecular Medicine, The Scripps Research Institute; [2]Immunology Team, Janssen Research and Development, LLC; [3]Department of Pediatrics, University of North Carolina; [4]University School of Medicine, Stanford University; [5]Department of Chemistry, University of Alberta*

CD22 is an inhibitory co-receptor of the B-cell receptor (BCR) and member of the sialic-acid binding immunoglobulin type-lectin (Siglec) family. Expression of CD22 on B-cells and most B-cell lymphomas has led to CD22 being considered an important therapeutic target for B-cell-mediated diseases. To better translate pre-clinic studies in mice to humans, an *in vivo* model where hCD22 is expressed on B-cells would be highly useful. Accordingly, we have developed a transgenic mouse expressing human CD22 (hCD22) and have examined the ability of hCD22 to functionally substitute for its murine counterpart in various aspects of B-cell biology. Expression of our hCD22 transgene on murine B-cells approaches the levels of hCD22 on human primary B-cells, and both hCD22 and mCD22 co-localize on the cell surface for B-cells that express both proteins. Murine B-cells expressing only hCD22 display equivalent calcium flux responses to anti-IgM as compared to WT B-cells. Moreover, hCD22 transgenic mice on a $mCD22^{-/-}$ background display equivalent numbers of marginal zone B-cells and antibody responses compared to WT mice. Furthermore, hCD22 transgenic mice, when challenged orally with crude peanut extract, develop the same level of allergic responses as WT mice. Surprisingly, B-cell homing to Peyer's patches was only partially rescued by hCD22 compared to $CD22^{-/-}$ B-cells. Finally, Siglec-engaging antigenic liposomes (STALs) displaying a hCD22 ligand prevent B-cell activation, induce apoptosis, and drive immunological tolerance *in vivo*. Our new hCD22 transgenic mouse should prove to be a valuable model for further investigating the function of hCD22 and for pre-clinical studies targeting hCD22.

### (187) Carbohydrate-neuroactive (CNH) strategy for non-invasive modulation of glycoconjugates of the central nervous system in vivo in mice

Surbhi Goswami[1], Asif Shajahan[1], Shubham Parashar[1], Hema Swasthi[1], Shanta Sen[2], Nagarajan Perumal[3] and Srinivasa Gopalan Sampathkumar[1]

*[1]Laboratory of Chemical Glycobiology (CGB), National Institute of Immunology (NII), New Delhi, India; [2]Central Mass Spectrometry Facility, National Institute of Immunology (NII), New Delhi, India; [3]Experimental Animal Facility (EAF), National Institute of Immunology (NII), New Delhi, India*

Glycosylation of proteins and lipids, sialylation in particular, plays vital functional roles in normal and healthy development of mammalian central nervous system (CNS) including the brain. However, very little literature exists on the connection between neurological diseases and neurodegenerative disorders and glycosylation. This is attributable to lack of probes for investigation of CNS glycosylation *in vivo*. During past two decades, the advent of metabolic glycan engineering (MGE) and bioorthogonal ligations has enabled tagging, imaging, and identification of glycoconjugates *in vivo*. Administration of 1,3,4,6-tera-O-acetyl-2-azidoacetylamino-2-deoxy-D-mannopyranose ($Ac_4$ManNAz) and 1,3,4,6-tetra-O-acetyl-2-propanoylamino-2-deoxy-D-mannopyranose ($Ac_4$ManNProp) in mice have been shown to be metabolized to corresponding neuraminic acids (NeuAz and NeuProp) in peripheral tissues such as heart, liver, and kidney. However, no MGE was found in brain which is attributable to poor transport of hexosamines across the blood-brain barrier. Recently, we have reported a carbohydrate-neuroactive hybrid (CNH) strategy for non-invasive delivery of hexosamine analogues to the brain (Shajahan, A., *et al. J. Am. Chem. Soc.*, **139**, 693–700 (2017)). CNH strategy envisages piggybacking of hexosamine analogues on neuroactive molecules that are known to readily cross BBB, in a prodrug-like design.

Our results showed that hybrids of 3,4,6-tri-O-acetyl-2-azidoacetylamino-2-deoxy-D-mannopyranose ($Ac_3$ManNAz) conjugated to nicotinate and valproate, but not $Ac_4$ManNAz, showed facile MGE of brain along with heart in mice upon intravenous administration followed by biotinylation using copper(I)-assisted azide-alkyne cycloaddition (CuAAC). CNH molecules exploit the abundant expression of various transport systems for vitamins and aminoacids at the BBB and enable non-invasive delivery of non-natural hexosamine analogues. Earlier reports have shown that 1,3,4,6-tetra-O-acetyl-2-butanoylamino-2-deoxy-D-mannopyranose ($Ac_4$ManNBut) inhibited biosynthesis of polysialic acid (polySia) on neural cell adhesion molecules (NCAM) *in vitro*. In order to design

probes for the modulation of polySia *in vivo*, hybrids of ManNBut conjugated to nicotinate were synthesized exploiting the CNH strategy. Levels of polySia were reduced significantly (as detected by western blotting using anti-polySia (12F8)) while the NCAM levels remained unaffected (as detected using anti-NCAM (1B11)) upon administration of $Ac_3$ManNBut-nicotinate, but not $Ac_4$ManNBut. This presentation will discuss our results on further investigations including the glycoproteomics identification of sialoglycoproteins in brain using mass spectrometry and their characterization. Considering the global initiatives for brain mapping and neurosciences, we believe that our non-invasive CNH strategy provides a facile access to study functions of glycosylation in brain during development and disease.

### (188) N-glycans in the antibody receptor CD16A exhibit carbohydrate-polypeptide contacts identified through solution NMR spectroscopy

Ganesh P. Subedi, Daniel J. Falconer and Adam W. Barb
*Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology. 2437 Pammel Drive. Molecular Biology Building, rm 4210. Iowa State University, Ames, IA 50011*

Asparagine-linked carbohydrates (N-glycans) are a common modification of eukaryotic proteins that confer multiple properties including the essential stabilization of therapeutic monoclonal antibodies. Here we present a rapid and efficient strategy to identify N-glycans that contact polypeptide residues and apply the method to profile the five N-glycans attached to the human antibody receptor CD16A (Fc gamma receptor IIIA). Human embryonic kidney 293 S cells expressed CD16A with [$^{13}C_U$]-labeled N-glycans using standard protein expression techniques and medium supplemented with 3 g/L [$^{13}C_U$]-glucose. Anomeric resonances on the protein-linked *N*-acetylglucosamine residue at the reducing end of the glycan are particularly well suited to studies of multiply-glycosylated N-glycoproteins because only one reducing end and nitrogen-linked residue is present in each N-glycan. Correlations between anomeric $^{1}H1$-$^{13}C1$ nuclei on the reducing end residue generate crosspeaks in a conventional 2d heteronuclear single quantum coherence NMR experiment that appear in a region of the spectrum devoid of other carbohydrate peaks or background protein signals. Two N-glycan peaks corresponding to the N45 and N162 N-glycans were dispersed from the rapidly averaged peaks corresponding to the N38, N74 and N169 N-glycans. We used a combination of NMR and 1 microseconds all-atom computational simulations to identify unexpected contacts between the N45 N-glycan and CD16A polypeptide residues.

### (189) Mouse IgG2b, 2c and human IgG1 antibodies have distinct structures and inequivalent function

Daniel J. Falconer and Adam W. Barb
*Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA*

Deglycosylating human IgG1 eliminates effector function, however, truncating the N297-glycan differently affects antibody subclasses. Kao et al (*Cell Reports*, 2015, 2376–2385) recently demonstrated truncating the mouse IgG2b N-glycan to a single GlcNAc residue eliminated effector function, but similar truncation of human IgG1 and mIgG2c retained function. This was surprising because the mIgG2b and mIgG2c heavy chains share 76% identity and are thought to be functionally equivalent to one another and human IgG1. Here we characterize how three amino acids contribute to functional diversification. The 2.0 Å structures of mouse IgG2c Fc in two N-glycoforms (with a single GlcNAc and the full N-glycan) displayed clear differences when compared to the mouse IgG2b Fc structure. The mouse Fcs form distinct inter-strand hydrogen bonding networks that stabilize the Fc gamma receptor-binding interface. Such networks are not observed in human IgG1 and mIgG2c forms more stabilizing interactions due to packing of P271 (found in both 2b and 2c) with L300 (I300 in 2b, Y300 in hIgG1 Fc). Solution NMR spectroscopy of the (1)GlcNAc residue confirmed the greater in N-glycan stabilization by mIgG2c. Switching three amino acid residues that form the distinct network between mIgG2b and 2c swapped the Fc gamma receptor IV binding and stabilization properties as judged by SPR and NMR, respectively. The Fc-stabilizing networks identified here highlight how antibody-receptor interactions are finely tuned and impact effector function. Furthermore, the networks are not conserved in hIgG1; demonstrating previously undescribed inter-species variability between homologous, but functionally inequivalent, antibodies.

### (190) Synthesis and analysis of rare sugar functionalized graphene oxide

Toru Mizuki[1,2], Keisuke Hirata[1], Yoshikata Nakajima[1,2], Takashi Uchida[1,2] and Toru Maekawa[1,2]
[1]*Graduate School of Interdisciplinary New Science, Toyo University;* [2]*Bio-Nano Electronics Research Centre, Toyo University*

Recently, graphene and graphene oxide (GO) has researched to apply for field of biology as scaffolding of culture and function materials, and reported that biocompatibility vary greatly depending on surface modification.

Graphene is the material of the planer sheet structure composed of carbon atoms. GO is a material which, is oxidized graphene, has various functional groups such as hydroxyl group, carboxyl group and epoxy group on the surface as side chains. There are a few contrasting functions in comparison between graphene and GO. Graphene is semiconductor but GO is insulator. Also, graphene has hydrophobic nature and GO has hydrophilic nature. Further, GO can be easily chemical modification but graphene is difficult. Reduced graphene oxide (RGO) has intermediate functions between graphene and GO, such as high conductivity, easily chemical modification, and great biocompatibility. RGO has

been applied for biosensor, cancer therapy, and organic solar cell. Common methods of reduction of GO are exposure treatment with hydrazine hydrate or heat treatment with electric furnace. However, the methods carry a lot of risk, so that green approaches are suggested for more safety and more ecological process (e.g. using microbial activity, vitamin C and polyphenol).

This study, we aim to synthesis rare sugar functionalized graphene oxide (RSfGO) by reducing GO using rare sugars that, are known as useful sugar, have many unique functions such as antioxidant action, inhibition of cancer cell growth, and function of control sugar level in the blood. Rare sugars are defines as monosaccharides and their derivatives being rarely in nature by International Society of Rare Sugars. D-psicose and D-allose are representative of rare sugars.

We attempted to reduce GO using some kinds of rare sugars. We mixed GO solution and rare sugars, and boiled it. After centrifuging, we washed precipitate with pure water for three times.

We analyzed surface state of prepared samples using Raman spectroscopy, x-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FT-IR), ultraviolet-visible absorption spectroscopy (UV-vis), and scanning electron microscope (SEM). Accordingly, G/D ratio of Raman spectrum graph and transition of absorbance spectrum of UV-vis suggested reduction of GO. We should obtain RSfGO that are created by reduction and modification of GO using rare sugars.

We analyzed cytotoxicity of the products with red blood cell (from sheep), bacteria (*Escherichia coli* JM 109 and *Bacillus subtilis* JCM1465) and human multiple myeloma cell (IM-9). It have been shown that RSfGO inhibited growth of *E.coli* and IM-9 cell, whereas RSfGO showed high biocompatibility to red blood cell.

### (191) AlphaScreen assays for detection of hyaluronan-protein binding

Mary K. Cowman[1], Xiayun Huang[1], Tannin A. Schmidt[2], Claire Shortt[3], Shivani Arora[1], Akira Asari[4] and Thorsten Kirsch[3]

*[1]Department of Chemical and Biomolecular Engineering, New York University Tandon School of Engineering, New York, NY USA; [2]Faculty of Kinesiology & Schulich School of Engineering, Centre for Bioengineering Research & Education, University of Calgary, Calgary, AB, Canada; [3]Department of Orthopedic Surgery, New York University School of Medicine, New York, NY USA; [4]Hyaluronan Research Institute, Inc., 2-5-8-1001, Nihonbashimuromachi, Chuo-ku, Tokyo, 1-3-0022 Japan*

We have employed AlphaScreen® (Amplified Luminescent Proximity Homogeneous Assay) technology for the quantitative study of interactions between hyaluronan (HA) and a group of specific HA-binding proteins. In AlphaScreen methods, two types of hydrogel-coated and chromophore-loaded latex nanobeads are employed. The proximity of the beads in solution is detected by excitation of the donor bead leading to the production of singlet oxygen, and chemiluminescence from the acceptor bead upon exposure to singlet oxygen. The donor bead can be modified with streptavidin, allowing the attachment of biotin-labeled HA. The acceptor bead can be modified with Ni(II), allowing attachment of specific recombinant HA-binding proteins (such as HABP; aggrecan G1-IGD-G2) with a His-tag. Alternative bead modification chemistries can be used for other attachment modes.

We have employed this method to develop a quantitative assay for HA. Competitive inhibition of the HA-HABP interaction by free unlabeled HA in solution is used for quantification. The assay is specific for HA, and not dependent on HA molecular mass above the decasaccharide. HA can be quantified over a concentration range of approximately 30–1600 ng/mL using 2.5 μL of sample, for a detectable mass range of approximately 0.08–4 ng HA. This sensitivity of the AlphaScreen assay is greater than existing ELISA-like methods, due to the small volume requirements. HA can be detected in biological fluids using the AlphaScreen assay, after removal of bound proteins from HA and dilution or removal of other interfering proteins and lipids. The new assay was used to determine the effect of IL-1β on the concentration of HA in conditioned medium from human articular chondrocytes. HA concentration increased with incubation time for both control and IL-1β treated cells, but the HA concentration was always higher for IL-1β treated cells.

The AlphaScreen platform has also been applied to test HA complexation with link protein, CD44, RHAMM, TSG-6 and TLR4. The HA-protein binding step was done in solution, then beads were added to detect the complexation. Multiple types of beads were used to capture protein targets with different tags. No binding was observed between TLR4 and HA. The binding of CD44 to HA was dependent on the site at which CD44 was tethered to the bead. If CD44 was labeled near its HA-binding region and then bound to a bead, steric hindrance from the bead prevents binding to HA. Labeling CD44 at a more distant site allowed HA binding.

AlphaScreen methods show promise for broader application to glycan-protein interactions.

### (192) Engineering of IgG Fc Glycosylation and the Relevant Activity Studies

Chung-Yi Wu[1,2], Chin-Wei Lin[1,2,3] and Chi-Huey Wong[1,2,3]

*[1]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan; [2]Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Academia Sinica, Taipei 115, Taiwan; [3]Department of Chemistry, National Taiwan University, Taipei 106, Taiwan*

Antibodies have been developed as therapeutic agents for the treatment of cancer, and infectious and inflammatory diseases. In addition to binding activity toward the target, antibodies also exhibit effector-mediated activities through the interaction of the Fc-glycan and the Fc-receptors on immune cells. In

order to identify the optimal glycan structures for individual antibodies with desired activity, we have developed an effective method to modify the Fc glycan structures to a homogeneous glycoform. In this study, it was found that the bi-antennary *N*-glycan structure with two terminal alpha-2,6-linked sialic acids is a common and optimized structure for the enhancement of antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and anti-inflammatory activities. Furthermore, both the pairing and the different modifications of Fc glycans, including the core-fucose, the branch, the length, and the sialylation linkage were investigated to supply a clear picture about the interactions with the FcrRIIIa/FcrRI and the corresponding effectors functions, including the ADCC and oxidative burst. The results indicate that the 1,6'-brach is more potent to increase the affinity towards FcrRIIIa/FcrRI than that of 1,3'-branch and such interactions intensify as the length of Fc glycans increase. Meanwhile, it was also illustrated that the 2,3-sialylation disrupts the aforementioned interaction and shows elevated dissociation rate constants whereas the 2,6-sialylation maintains the affinity compared to the non-sialylated counterpart. Among the various antibodies with different Fc glycan modifications, the adverse effect of core-fucosylation on the affinity exceeds the others in the case of anti-FcrRIIIA but lessens for FcrRI. Moreover, not only the affinity but also the threshold for activating FcrRIIIA was found to be mediated by various Fc glycans. Interestingly, the Fab/Fc interdomain interaction and the minor adjustments of Fab binding by the Fc glycans were observed as well. The affinity and the threshold for FcrRs activation might relate to the efficiency of antigen uptake whereas the fine-tuning of the Fab recognition could lead to the distinct footprint for presenting to T cells after the proteolytic processing. Based on the results in this study and previous reports, an alternative affinity maturation mediated by the Fc glycosylation was implied.

### (193) Determination of the minimum enzymatic domain of keratanase II

Tomoya O. Akama[1], Toshisuke Kawasaki[2] and Tomoyuki Nakamura[1]

*[1]Kansai Medical University; [2]RItsumeikan University*

Keratanase II is an endo-GlcNAcase that hydrolyzes keratan sulfate glycosaminoglycans (KS-GAGs). Currently two bacteria strains (Bacillus sp. Ks36 and Bacillus circulans KsT202) have been reported to produce keratanase II, and keratanase II cDNA has been cloned from Bacillus circulans KsT202 as a 200 kDa membrane bound enzyme (Yamagishi K., et al., J. Biol. Chem. 278, 25766–72, 2003). Steward et al. reported that truncated keratanase II, which lacks a C-terminal membrane bound region, also hydrolyzes KS-GAGs and demonstrated that the recombinant truncated enzyme can be used to quantify urine KS-GAGs for diagnosis of mucopolysaccharidosis IVA (Morquio A syndrome) (Clin. Biochem. 48, 796–802, 2015). In this study, we analyzed enzymatic activity of keratanase II deletion mutants, and identified a 110 kDa polypeptide as the minimum enzymatic domain of keratanase II. We also purified a recombinant 110 kDa keratanase II and characterized its digesting activity of KS-GAGs.

### (194) High throughput comprehensive analysis of glycoproteins through Tool for Rapid Analysis of glycopeptide by Permethylation (TRAP) method

Asif Shajahan, Nitin T. Supekar, Christian Heiss, Ian Black and Parastoo Azadi

*Complex Carbohydrate Research Center, University of Georgia, Athens, GA - 30602*

Developing a comprehensive and universal tool for the automated analysis of glycopeptides through their tandem mass spectrometry and data processing is presently a challenging task. We have developed a method, Tool for Rapid Analysis of glycopeptide by Permethylation (TRAP), that has the potential to simplify glycoprotein analysis by integrating glycan sequencing and glycopeptide analysis in a single experiment. We have demonstrated that through this approach glycosidic linkages, composition, glycan sequencing, glycosylation site mapping and sequencing of peptide moiety of glycopeptides of glycoproteins from both prokaryotes and eukaryotes could be characterized. Here we present the application of TRAP in a high throughput permethylation analysis, which is easy to use, robust, suitable for large as well as small sample sets and also cost-effective. We have developed a permethylation protocol which can be automated and suitable for reliable permethylation of glycopeptides from the protease digest of glycoproteins. Our preliminary studies on glycopeptides from bovine RNAse B digest indicates that using optimum reaction conditions and sample purification improves the sensitivity and throughput of permethylation reactions in microscale. This strategy of glycoprotein characterization would enable convenient, rapid, and reliable high-throughput screening of protein glycosylation and can be used for the development of a cost-effective kit for glycosylated biomarker discoveries. In addition to this, we have optimized an LC-MS/MS platform for the analysis of glycopeptides processed through TRAP, for the analysis of glycoforms from complex glycoproteins. Our results on the analysis of standard glycoproteins such as bovine RNase B, human transferrin, and bovine fetuin indicate that TRAP hyphened with LC-MS/MS enables precise characterization of isomers of each glycoforms at each glycosylation sites of complex glycoprotein mixtures.

### (195) Novel Sialoglycan Lectenz® Reagents

Sheng-Cheng Wu[1], J. Christopher Cooper[1], Mallory K. Paul[1], Ziad M. Eletr[1], Shani L. Ben-Arye[2], Vered Padler-Karavani[2], Kausar N. Samli[1] and Robert J. Woods[3,1]

*[1]Lectenz® Bio, Athens, Georgia, United States; [2]Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel; [3]Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, United States*

Glycans have distinct properties that make them appealing as disease biomarker targets. Firstly, their location on cell surfaces makes them the first point of contact for cellular interactions, and thus they are crucial in the control of normal metabolic processes, and conversely, they function as pathogen adhesion receptors. Secondly, specific glycan structures that are not present, or are in low amounts in normal state, proliferate or alter their sequence in disease states. Changes in the normal levels of glycan structures, such as terminal sialic acid, may be markers of diseases such as cancer. New highly specific reagents are essential to overcome current limitations in the discovery and exploitation of disease-related glycans.

Lectenz® Bio is engineering glycan-processing enzymes into catalytically inactive, high affinity glycan binding reagents with tunable specificities. These novel lectin-like, enzyme-derived reagents called Lectenz® are being developed for a variety of glycan detection and enrichment applications including affinity chromatography, Western blot, and immunohistochemistry. The conversion of such enzymes into affinity reagents is facilitated by computationally-guided directed evolution. Using site-directed mutagenesis and yeast display selection of a site-saturation mutagenesis library, multiple Lectenz® candidates can be identified.

Here we present two novel sialic acid recognizing Lectenz® engineered from a sialidase enzyme: 1) the pan-specific sialic acid reagent, Sia-PS1 Lectenz®, which recognizes sialic acid in a linkage independent manner; and 2) the Sia-3S1 Lectenz®, which is specific for α2,3-linked sialoglycans. The data demonstrate that the Sia-PS1 Lectenz® reagent recognizes glycans terminating in α2,3-, α2,6-, and α2,8-linked sialic acid sequences, but not Gal-terminating sequences. With the Sia-3S1 Lectenz®, we demonstrate binding to Neu5Acα2,3Galβ1,4Glc, but not Neu5Acα2,6Galβ1,4Glc or Galβ1,4Glc.

## (196) GALECTIN-1/MALTOSE BINDING PROTEIN FUSION MOLECULE: A PROMISSING TOOL TO DESING A ELETROCHEMICAL LECTIN-BASED BIOSENSOR

Pâmela O M Gomes[1], Thais C. De Leo[2], Marina R. Batistuti[1], Richard D. Cummings[3], Marcelo Dias-Baruffi[2] and Zeki Naal[2]

*[1]Faculty of Philosophy Sciences and Letters of Ribeirão Preto - University of São Paulo - Brazil.; [2]School of Pharmaceutical Sciences of Ribeirão Preto - University of São Paulo – Brazil.; [3]Department of Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02115, United States of America*

The integration of various areas of science is critical for the development of new methods on bioanalytical technology, which improves accuracy, sensitivity, and operational simplicity with low cost. Accordingly, the association between electrochemical approaches, genetic/molecular engineering, and lectin-based biosensors is a very attractive strategy for the detection and quantification of bio-analytes. In this work, we described the expression, purification and partial characterization of a novel chimeric-lectin composed by Galectin-1 (Gal-1), a multifunctional β-galactoside-binding protein, and Maltose Binding Protein (MBP). The soluble and homogenous preparation of MBP-Gal-1 was obtained by affinity chromatography on lactosyl-agarose and analyzed by SDS-PAGE, MALDI-TOF/TOF, Western-blot, and Dynamic Light Scattering (DLS). Hemagglutination assays were used to evaluate the MBP-Gal-1 lectin activity. Also, the electrochemical behavior of this biomolecule (MBP-Gal-1) was reported using Electrochemical Impedance Spectroscopy (EIS). For EIS analyses, MBP-Gal-1 was immobilized onto an electrode surface modified with a redox polymer, obtained from oxidation of N-(3-pyrrol-1-ylpropyl)-4,4'-bipyridine (PPB), which interacts with the MBP. The pure MBP-Gal-1 presented molecular mass of 57,83 kDa and its lectin activity was inhibited by lactose. DLS analyses indicated that MBPGal-1 preparation shows a single monodisperse (99.8%) peak with hydrodynamic radius of $4 \pm 1{,}26$ nm. Preliminary data on EIS suggested that lactose interactions with MBP-Gal-1 has a better impedance change compared to lactose-Gal-1 interaction. These results suggest that MBP-Gal-1 could be a promising lectin-based biosensor for electrochemical platform to evaluate the interactions between Gal-1 and different targets. Finally, this system could provide an oriented immobilization of MBP-Gal-1 on surface of PPB-polymers with Gal-1 preserved lectin activity for bioanalytical assays.

## (197) Characterization of a Novel Mouse Strain Expressing Human Siglec-8 Only on Eosinophils

Jeremy A. O'Sullivan[1], Yadong Wei[2], Daniela J. Carroll[1], Liliana Moreno-Vinasco[1], Yun Cao[1], Fengrui Zhang[2], James J. Lee[3], Zhou Zhu[2] and Bruce S. Bochner[1]

*[1]Division of Allergy and Immunology, Department of Medicine, Feinberg School of Medicine, Northwestern University; [2]Department of Medicine, Yale University School of Medicine; [3]Division of Pulmonary Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic Arizona*

Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 is a human cell surface protein expressed exclusively on eosinophils, mast cells, and basophils that, when engaged, induces eosinophil apoptosis and inhibits mast cell mediator release. This makes Siglec-8 a promising therapeutic target to treat diseases involving these cell types. However, pre-clinical studies of the effects of Siglec-8 targeting in vivo are lacking due to the fact that this protein is only found in humans, apes, and some monkeys. Therefore, we have developed a new Siglec-8 knock-in mouse strain.

The *SIGLEC8* gene and an upstream loxP-flanked STOP cassette were introduced by homologous recombination into the *Rosa26* locus. When crossed with mice in which Cre recombinase expression is driven by the eosinophil peroxidase promoter (*eoCre*), the STOP cassette is removed and

Siglec-8 is expressed in these cells. To initially characterize this new strain, we examined Siglec-8 surface expression, endocytosis, and signaling in mouse eosinophils from various tissues and those differentiated in vitro from bone marrow as well as Siglec-8 effects in an allergic lung inflammation model and in the context of IL-5 stimulation.

We confirm that the Siglec-8 protein is expressed on the surface of mature eosinophils and only on eosinophils in multiple tissue compartments at levels comparable to those seen on human blood eosinophils. No changes in baseline blood or tissue eosinophil numbers were evident in these mice compared to littermate controls. Using eosinophils derived from bone marrow of these mice, we demonstrate that, during maturation, Siglec-8 surface expression occurs after Siglec-F but before the appearance of the late eosinophil developmental marker CCR3, consistent with the timing of eosinophil peroxidase expression during eosinophil development. In response to antibody ligation, Siglec-8 is endocytosed more rapidly than Siglec-F, and this ligation alters the phosphotyrosine profile of these cells, indicative of ligand-induced signaling. Employing a model of ovalbumin sensitization and airway challenge, we show that this Siglec-8 knock-in mouse strain generates a similar pattern of allergic lung inflammation when compared to littermate controls. Eosinophils from the spleens of IL5Tg mice, in which IL-5 expression is driven in T cells, undergo cell death when treated with anti-Siglec-8 mAb in vitro, demonstrating that the receptor is functional on these cells.

This new mouse strain should be a valuable tool to investigate Siglec-8 targeting in vivo in a variety of eosinophilic disease settings. By enabling more facile manipulation compared to human eosinophils, this mouse strain may also shed new light on other aspects of Siglec-8 biology.

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### (198) Identification of carbohydrate-mimetic D-amino acid peptides by peptide-display library screening as chemotherapy for malignant brain tumors

Motohiro Nonaka[1,2], Misa S. Anekoji[2], Tomoya O. Akama[3], Jun Nakayama[4] and Michiko N. Fukuda[1,2]

*[1]Advanced Industrial Science and Technology; [2]Sanford-Burnham-Prebys Medical Discovery Institute; [3]Kansai Medical University; [4]Shinshu University School of Medicine*

Previously, we identified a series of carbohydrate-mimetic peptides by screening peptide-display phage libraries targeting anti-carbohydrate antibodies. One peptide designated IF7 bound to the N-terminus of annexin 1 (Anxa1). Anxa1 is expressed on the surface of vascular cells specifically in malignant tumors. We found that an IF7-conjugated anti-cancer drug injected intravenously into tumor-bearing mice efficiently suppressed tumor growth without side effects (Hatakeyama et al., Proc. Natl. Acad. Sci. USA 2011). In brain tumor-bearing mice, IF7-injected intravenously was transported through endothelial cells and released to the stroma in brain, suggesting that an IF7-conjugated drug can overcome blood-brain barrier. Indeed IF7-conjugated to an anti-cancer drug effectively suppressed brain tumors in vivo in mouse. To improve IF7 therapeutic efficacy, we searched for novel peptides made of D-type amino acids, taking advantage of the fact that the Anxa1 N-terminus is sufficient for IF7 binding. Following screening of a phage library with a synthetic Anxa1 N-terminal domain peptide comprised of D-amino acids, we identified peptides that bound to Anxa1 protein. When fluorescence-tagged, someof those D-peptides targeted brain tumors in vivo in mouse. One of the D-peptide conjugated with anti-cancer drug, when orally administrated, suppressed brain tumor growth, This work suggests that carbohydrate-mimetic D-peptides could serve as orally-administered anti-cancer therapeutics.

### (199) Development of high affinity and immunomodulatory ligands of C-type lectin receptor langerin by oligomerizing a keratan sulfate disaccharide L4

Fumi Ota[1], Yasuhiko Kizuka[1], Tetsuya Hirayama[2], Yoshiki Yamaguchi[3], Masahiro Nagata[4], Hendra S. Ismanto[4], Bernd Lepenies[5], Jonas Aretz[6,7], Christoph Rademacher[6,7], Peter H. Seeberger[6,7], Takashi Angata[8], Shinobu Kitazume[1], Keiichi Yoshida[1], Tomoko Betsuyaku[9], Kozui Kida[10], Sho Yamasaki[4,11,12] and Naoyuki Taniguchi[1]

*[1]Disease Glycomics Team, RIKEN, Japan; [2]Central Research Laboratories, Seikagaku Corporation, Japan; [3]Structural Glycobiology Team, RIKEN; [4]Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, Japan; [5]Research Center for Emerging Infections and Zoonoses (RIZ), University of Veterinary Medicine Hannover, Germany; [6]Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Germany; [7]Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Germany; [8]Institute of Biological Chemistry, Academia Sinica, Taiwan; [9]Division of Pulmonary Medicine, Department of Medicine, Keio University, Japan; [10]The Respiratory Care Clinic, Nippon Medical School, Japan; [11]Laboratory of Molecular Immunology, Immunology Frontier Research Center, Osaka University, Japan; [12]Division of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, Japan*

Langerin is a C-type lectin receptor expressed in a subset of dendritic cells and binds to glycan ligands for pathogen capture and clearance. Previous unbiased studies using glycan arrays showed that langerin unusually binds to 6-sulfated galactose, a keratan sulfate (KS) component. However, details and biological outcomes of this interaction have not been studied. We recently reported that a KS disaccharide L4, which includes 6-sulfated galactose, exhibited therapeutic and anti-inflammatory activity in COPD mouse models (Gao et al., Am. J. Physiol. Cell. Mol. Physiol., 2017). This suggests a possibility that L4-

related compounds can be used for detailed characterization of langerin-ligand interaction and its therapeutic application.

In this study, by using a series of KS disaccharides with different sulfation patterns, we first show that the langerin binding requires the 6-sulfation of galactose and that trimeric form of langerin has higher affinity than the monomeric form. To next design a new high affinity ligand, we synthesized trimeric and polymeric L4 derivatives, and showed that these compounds exhibited over 1,000-fold higher affinity to langerin than free L4 in ELISA assays. In addition, the polymeric L4 effectively transduced langerin signaling in a reporter cell system, and the trimeric L4 suppressed lung inflammation in an emphysema mouse model. These results suggest that oligomeric L4 derivatives are powerful tools for clarifying the langerin functions and for the development of new drugs for inflammatory diseases.

## (200) Automated Identification and Subsequent Relative Quantitation of Glycans Using Stable Isotope Labels by MS with SimGlycan software

Ningombam Sanjib Meitei[1,2]
[1]*PREMIER Biosoft, Indore, India;* [2]*PREMIER Biosoft, Palo Alto, US*

Recent trends in glycomics research show rapid growth of innovation related to accurate mass spectrometry based quantitative analysis of glycans. Stable isotope labels facilitate accurate identification, and subsequent quantification of glycans using LC-MS or ESI-MS workflows.

There are two major types of stable isotope labeling approaches reported in literature namely, (1) mass shift e.g., isotopic labeling using $^{18}O/^{16}O$-Water [1] or $^{12}$[C6]-2-AA)/$^{13}$[C6]-2-AA [2], and (2) isobaric tags e.g., aminoxyTMT6, and QUANTITY [3]. The mass shift method employs chemically similar but isotopically differentiated tags to derivatize glycan samples, so that they can be distinguished and quantitatively compared by MS. Here, quantitation of glycans in samples is achieved in MS1. Another recently reported mass shift technique is the Dual Reactions for Analytical Glycomics (DRAG) [4], to quantify both neutral and sialylated glycans simultaneously and comparatively. While, in isobaric tags methods, quantification relies on reporter ions generated in MS2 or MS3.

Unlike in proteomics, there is no adequate software tools to exploit the recently developed above-mentioned glycomics methods. To the best of our knowledge, there has not been any software solution that can perform automated data analysis from the DRAG method. We have developed SimGlycan software modules to streamline the analysis of data generated by above qualitative and quantitative methods.

### References

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