(3) Recycling of Golgi Glycosyltransferases Requires Direct Binding to Coatomer

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The glycosyltransferases of the mammalian Golgi complex must recycle between the stacked cisternae of that organelle to maintain their proper steady state localization. This trafficking is mediated by COPI-coated vesicles, but how the glycosyltransferases are incorporated into these transport vesicles is poorly understood. Here we show that the N-terminal cytoplasmic tails of a number of cis Golgi glycosyltransferases which share a Ø-(K/R)-X-L-X-(K/R) sequence bind directly to the delta and zeta subunits of COPI. Mutations of this N-tail motif impair binding to the COPI subunits, leading to mislocalization of the transferases to lysosomes. The physiological importance of these interactions is illustrated by mucolipidosis III patients with missense mutations in the N-tail of GlcNAc-1-phosphotransferase that cause the transferase to be rapidly degraded in lysosomes. These studies establish that direct binding of the N-tails of mammalian cis Golgi glycosyltransferases with COPI subunits is essential for recycling within the Golgi.

(4) Structural basis for nucleotide sugar transport across the ER and Golgi membranes

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Glycosylation is a fundamental cellular process that in eukaryotes occurs in the lumen of both the Golgi apparatus and endoplasmic reticulum. Nucleotide sugar transporters (NSTs) are an essential component of the glyscosylation pathway, providing the diverse range of substrates required for the glycosyltransferases. NSTs are linked to several developmental and immune disorders in humans and in pathogenic microbes play an important role in virulence. How NSTs recognise and transport activated monosaccharides however is currently unclear. In this talk I will present the crystal structure of the GDP-mannose transporter Vrg4, in both the substrate free and bound states. A hitherto unobserved requirement for short chain lipids in activating the transporter supports a model for regulation within the highly dynamic membranes of the Golgi apparatus. Our results provide a structural basis for understanding nucleotide sugar recognition and provide insights into the transport and regulatory mechanism for the SLC35 family of intracellular transporters.

(5) GALA: a glycosylation programme controlling tumor growth and tissue invasion

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GalNac type O-glycosylation, contrary to N-glycosylation, is typically started in the Golgi apparatus. The polypeptide N-acetyl-Galactosamyl transferases (GALNTs) are initiating the O-glycosylation pathway by generating the Tn glycan. GALNTs act on thousands of sites in cell surface and secreted proteins. GALNTs can be relocated from Golgi to the ER upon Src activation, a process we called GALA. We find that GALA stimulates the glycosylation of about 20% of detectable glycosites. Roughly 200 proteins and 800 glycosites are strongly affected. Many of these proteins have been implicated in tumorigenesis. One of them is the metalloprotease MMP14, whose glycosylation strongly increases activity. GALA also affects the ER resident protein Calnexin, which we find also required for ECM degradation. In a mouse model of liver tumor, stimulating GALA leads to a strong acceleration of tumor progression, while inhibiting it blocks it. In sum, we propose that GALA is a glycosylation program that controls matrix degradation and tissue invasion and is required for tissue remodelling during tumor growth.

(6) COG and GARP protein complexes are essential for the maintenance of Golgi glycosylation machinery and for endosomal to Golgi vesicular trafficking

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The Conserved Oligomeric Golgi (COG) complex controls membrane trafficking and ensures Golgi homeostasis by orchestrating retrograde vesicle trafficking within the Golgi. Human COG defects lead to severe multi-systemic diseases known as COG-Congenital Disorders of Glycosylation (COG-CDG). The Golgi Associated Retrograde Protein (GARP) complex regulates retrograde transport from endosomes to the trans-Golgi network. The CRISPR approach was utilized to generate HEK293T and HeLa cells deficient for individual COG and GARP subunits as well as for several key double knock-down (DKO) combinations.

COG KO cells show extensive defects in Golgi morphology, retrograde trafficking, sorting, protein and lipid glycosylation. COG KO defects were completely rescued by stable expression of COG-GFP constructs. Surprisingly all COG KO cells also display drastically enlarged endo-lysosomal structures (EELSs) and transcriptional upregulation of cholesterol synthesis. EELSs are cholesterol-rich, acidic and positive for Rab7a, Rab9a and Lamp2a. To gain better understanding of COG-CDGs we used lectin blotting, flow cytometry, EM and superresolution microscopy to compare COG KO cells to cells deficient to two key enzymes, Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase (MGAT1) and UDP-glucose 4-epimerase (GALE), which control Golgi N- and O-glycosylation. The CRISPR approach was utilized to generate a HEK293T MGAT KO, GALE KO and MGAT/GALE double KO cell lines. While all KO cells share similar defects in glycosylation, these defects only account for a small fraction of COG KO phenotypes. A complete block in both

N- and O- Golgi glycosylation achieved in MGAT1/GALE double KO cells was also insufficient to completely match COG-deficient phenotypes. Glycosylation deficiencies were not associated with the fragmented Golgi, EELSs, enhanced secretion of unglycosylated proteins, defective sorting of Cathepsin D, or delayed retrograde trafficking indicating that these phenotypes are not due to hypoglycosylation, but to other specific roles of the COG complex. Importantly, COG deficiency specific phenotypes, including accumulation of EELSs and Cathepsin D sorting defects, were confirmed in COG7-CDG patient fibroblasts proving the human disease relevance of our CRISPR KO findings.

GARP KO cells show defects in retrograde trafficking and, surprisingly, in both N- and O-glycosylation. Glycosylation and trafficking defects were even more severe in COG/GARP DKO cells, while the enlargement of late endosomal compartment was partially suppressed, indicating that the GARP activity is required for formation of aberrant endosomal compartment.

Stability of both cis/medial (MGAT1) and trans-Golgi (B4GALT1 and ST6GAL1) enzymes was compromised in both COG and GARP KO cells, indicating that these complexes are essential for the maintenance of Golgi glycosylation machinery. To gain a better understanding of Golgi enzyme fate in COG and GARP deficient cells we have utilized a RUSH pulse-chase approach, investigating intracellular trafficking and degradation of newly synthesized B4GalT1 and MAN2A1.

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(7) In vivo kinetic analysis of the heterogeneous N-glycan-processing

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Site-specific glycan profiles are dependent of various parameters including expression levels of glycosyltransferases and hydrolases, sugar-donor concentration, glycan availability and its interaction with the glycoprotein surface, but also the time of exposure to the different carbohydrate-active enzymes and the sequence of the maturation events in the ER and Golgi. Compartmentalization of the glycosylation processes promotes competition of different enzymes for the same substrate and short exposure time of the glycan to the processing enzymes. We previously established that the model protein PDI (protein disulfide isomerase) carries five sites of glycosylation which are differentially processed, and that this site-specific glycosylation is dependent of the glycan-to glycoprotein interaction.

In order to study how the different glycan processing steps and the site-specific glycosylation are correlated in vivo, we combined SILAC and LC-MS-MS approaches. For our studies, we used CHO cells stably expressing the model protein. Cells were cultivated in regular medium, and then shifted to 15 N arginine- and lysine-containing medium. Cells were sampled every 15 minutes, intracellular and secreted PDI was purified and the site-specific glycosylation was analyzed by mass spectrometry in order to follow the appearance of heavy neo-synthesized glycoprotein. We followed N-glycan processing by monitoring 42 different structures on five different glycopeptides. The ratio between the heavy glycopeptide of each glycoform and the total amount of the glycoform (heavy+light) on a specific peptide was calculated and used to mathematically model the secretion, the enzyme distribution and the processing events in the site-specific N-glycosylation pathway.

Our results allow to order the processing steps of the glycosylation pathway in a time-resolved manner, and reveal different kinetics of appearance of the same glycoform on different sites of the glycoprotein, confirming the site-specific effect on the glycan accessibility and its processing.

(8) Structure and mechanism of eukaryotic oligosaccharyltransferase complex

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Eukaryotic oligosaccharyltransferase (OST) is a multi-subunit protein complex embedded in the membrane of theendoplasmic reticulum (ER). It catalyzes the *en bloc* transfer of a high-mannose oligosaccharide from a dolichol-pyrophosphate carrier onto asparagines located in glycosylation sequons (sequence N-X-S/T) of secretory proteins.

Using single-particle cryo-electron microscopy, we have determined the structure of a fungal octa-subunit OST complex reconstituted in lipidic nanodiscs. The activity of reconstituted *Saccharomyces cerevisiae* OSTwas assessed using an *in vitro* glycosylation assay with synthetic LLO and peptide substrates. The structure, determined at 3.31 Å resolution, revealed the arrangement of the eight subunits and suggested that eukaryotic OST complexes have a conserved architecture. Using the crystal structure of the bacterial homolog of the catalytic STT3 subunit, the PglB protein of *Campylobacter lari*, features critical for catalytic activity and substrate recognition could be identified on the surface of the STT3 subunit. The cryo-EM structure not only revealed ordered lipid molecules, but also a large N-glycan attached to a conserved Asn residue within STT3. This glycan lines a cavity that may serve as the glycan-binding pocket for the donor LLO substrate.

By docking the structure of yeast OST into previously determined tomography maps, insight into distinct features of OST complexes either involved in co-translocational N-glycosylation (OST complexes associated with the translocon) or in post-translocational N-glycosylation (standalone OST complexes containing a subunit with redox chaperone) could be gained. Our results not only reveal the architecture of the OST complex and suggests roles for the non-catalytic subunits, they also suggest mechanisms by which eukaryotic OST complexes stimulate N-glycosylation over folding of large numbers of secretory proteins.

(9) Structural mechanism of the eukaryotic oligosaccharyl transferase complex

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N-glycosylation is one of the most dominant modifications of eukaryotic proteins. It is catalyzed by oligosaccharyl transferase (OST), an eight-subunit membrane protein complex in ER membrane. OST transfers the oligosaccharide from a lipid-linked donor (LLO) to the Asn-Xaa-Ser/Thr sequon of a nascent polypeptide, usually co-translationally by partnering with the ribosome and translocon. We recently determined a high-resolution cryo-EM structure of the yeast OST complex, with samples purified in a relatively mild detergent digitonin from the endogenous source. In my presentation, I will describe the molecular mechanism of eukaryotic OST and its potential interaction with the translocon, as gleaned from the atomic model of the OST complex.

(10) Structural mechanisms underlying the ER quality control associated with glucose tagging

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N-linked oligosaccharides attached to proteins act as tags for glycoprotein quality control, ensuring their appropriate folding and trafficking in cells. Monoglucosylated glycoforms are the hallmarks of incompletely folded glycoproteins inthe protein quality-control system, in which glucosidase II (GII) and UDP-glucose:glycoprotein glucosyltransferase (UGGT) are respectively responsible for glucose trimming and attachment.Here, wepresent integrative structural data of these key enzymes as well as substrate*N*-linked oligosaccharides exhibiting flexible structures, as revealed by applying a series of biophysical techniques.

RegardingGII, we solvedthe crystal structures of its catalytic subunit complexed with two different glucosyl ligands, providing a structural basis for substrate-binding modes in the two-step reactions.Our results suggest that the two-step glucose trimming reactions catalyzed in the active-site pocket do not successively proceed by virtue of its gourd-shaped architecture.Presumably,glycoproteins thus have a time window between the first and second step of deglucosylation for chaperone-mediated folding to occur, mediated by the calnexin-containing folding complex that specifically recognizes theterminal monoglucose residue of the*N*-glycan tags.

We also characterized dynamic conformation of a high-mannose-type dodecasaccharide with a terminal glucose residue (GM9), i.e., GII substrate or UGGT product. TheGM9oligosaccharidewas prepared by our developed chemoenzymatic technique, which uses^{13}C labelling and lanthanide tagging to detect conformation-dependent paramagnetic effects by NMR spectroscopy. The NMR-validated molecular dynamics simulation produced the dynamic conformational ensemble of theGM9. Comparison of our data with previously reported crystallographic result indicates that the lectin binding to its target oligosaccharides involves an induced-fit mechanismwith an entropic penalty. It is possible that this negatively-regulated glycan interaction is importantin the quality-control systemfor selective dual recognition of unfolded glycoproteins carrying GM9 in addition tounstructured polypeptide chains.

As for UGGT, our crystallographic data demonstrate that the folding sensor region is composed of four thioredoxin-like domains followed by a β-rich domain, which are arranged into a C-shaped structure with a large central cavity. Furthermore, small-angle X-ray scattering, cryo-electron microscopy and high-speed atomic force microscopy have demonstrated that UGGT has a flexible modular structure in which the smaller catalytic domain is tethered to the larger folding sensor region with variable spatial arrangements. These findings provide structural insights into the working mechanism whereby UGGT operates as a folding-sensor against a variety of glycoprotein substrates through its flexible modular structure possessing extended hydrophobic surfaces for the recognition of unfolded substrates.

(11) Structural and mechanistic insights into GalNAc-type O-glycosylation

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The polypeptide GalNAc-transferases (GalNAc-Ts), that initiate mucin-type O-glycosylation, consist of a catalytic and a lectin domain connected by a flexible linker. In addition to recognizing polypeptide sequence, the GalNAc-Ts exhibit unique long-range N- and/or C-terminal prior glycosylation (GalNAc-O-Ser/Thr) preferences modulated by the lectin domain. Here we report studies on GalNAc-T4 that reveal the origins of its unique N-terminal long-range glycopeptide specificity, which is the opposite of GalNAc-T2. The GalNAc-T4 structure bound to a monoglycopeptide shows that the GalNAc-binding site of its lectin domain is rotated relative to the homologous GalNAc-T2 structure, explaining their different long-range preferences. Kinetics and molecular dynamics simulations on several GalNAc-T2 flexible linker constructs show altered remote prior glycosylation preferences, confirming that the flexible linker dictates the rotation of the lectin domain, thus modulating the GalNAc-Ts' long-range preferences. This work for the first time

provides the structural basis for the different remote prior glycosylation preferences of the GalNAc-Ts.

(12) The structural basis for Protein-O-glycosylation of EGF-like and TSR domains

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Epidermal Growth Factor-like (EGF-like) and Thrombospondin Repeat (TSR) domains are small disulfide-containing protein domains that are ~40-60 amino acids in length. These domains are often tandemly repeated in the proteins that contain them and they are found in a number of eukaryotic cell surface and secreted proteins that mediate processes ranging from cell signaling to blood coagulation. Most notable, is the preponderance of EGF-like domains found in the Notch Receptors and their ligands.

Both EGF-like and TSR domains are found to be O-glycosylated by protein-O-glycosyltransferases that are specific for them. Protein O-fucosyltransferase 1 (POFUT1), for example, fucosylates only EGF-like domains, while Protein O-fucosyltransferase 2 (POFUT2) is specific for TSRs. Not all EGF-like and TSR domains are fucosylated and the specificity shown by these enzymes is mediated, in part, by the recognition of short sequence motifs that contain the Ser/Thr residues fucosylated in those domains that are. Two other protein-O-glycosyltransferases, RUMI/Protein O-glucosyltransferase 1 (POGLUT1) and EGF domain-specific O-linked N-acetylglucosamine transferase (EOGT), each recognize a different sequence motif and are responsible for transferring glucose/xylose and N-acetylglucosamine to EGF-like domains. In all cases, these protein-O-glycosyltransferases are ER-resident and considerable evidence suggests that their action is important in quality control and/or the trafficking of their glycosylated substrates out of the ER.

We have determined the x-ray crystal structures of POFUT1, POFUT2 and POGLUT1 in complexes with several natural and synthetic EGF-like and TSR domains. These structures have provided important insights into the role played by the sequence motif for glycosylation, as well as overall shape complementarity between enzyme and substrate as it pertains to substrate recognition and catalysis. In addition, these complexes explain how these enzymes have evolved to tolerate the vast sequence diversity shown by their EGF-like and TSR substrates.

Based on database sequence analysis, we have also analyzed the occurrence of POFUT1 and the subset of EGF-like domains typical of Notch and its receptors for all the organisms where whole genome sequences exist. Without exception, we found that both POFUT1 and these EGF-like domains are present in all animal species and their closest protist ancestors, an observation suggesting that POFUT1-mediated fucosylation has played a critical role in animal evolution.

(13) Origin of acceptor specificity in GT-A fold GlcNAc transferases

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Cell surface glycans display incredible diversity, and are essential for cell signaling, cell adhesion and host-pathogen interactions. Glycan structures are not encoded by specific templates, but instead are derived from trimming, branching and extension reactions that occur in the Golgi. These biosynthetic pathways compete for the glycan acceptor substrate, and often the product one enzyme will prevent action of others. How these glycoenzymes recognize specific acceptor substrates is important for understanding glycan diversity. The enzyme N-acetylglucosaminyltransferase II (MGAT2) produces the second GlcNAcβ1,2- branch on the trimannosyl glycan core of the precursor to complex-type N-glycans using UDP-GlcNAc as a sugar donor. Here, we describe the crystal structure of MGAT2 in complex with the GlcNAc- Man3GlcNAc2-Asn acceptor. The complex shows that acceptor specificity in MGAT2 originates from both active site interactions with the Man-α1,6-monosaccharide acceptor and an exosite that binds the GlcNAc-β1,2Man-α1,3Manβ- substrate "recognition arm." The exosite is strikingly similar to that observed in the unrelated Golgi enzyme α-mannosidase II, which acts immediately before MGAT2 in the N-linked glycan biosynthetic pathway. Comparisons of MGAT2 with other GT-A fold glycosyltransferases reveals a modular architecture for achieving the donor nucleotide and acceptor substrate specificity. The donor nucleotide module is formed by amino acid interactions within the GT-A fold. In contrast, the acceptor template module is built from structural elements inserted into the conserved GT-A fold. This likely contributes to the evolution of glycan diversity. Because the acceptor template module is formed from elements inserted into the stable GT-A scaffold, the evolution of acceptor specificity is decoupled from the protein folding constraint and can evolve more rapidly.

(14) The structure of GalNAc-T12 reveals the molecular basis of its substrate recognition mode

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Polypeptide *N*-acetylgalactosaminyl transferases (GalNAc-Ts) initiate mucin type O-glycosylation by catalyzing the transfer of GalNAc to a Ser or Thr on a substrate. The 20 human GalNAc-T isoforms contain a catalytic and lectin domain that are involved in substrate binding. However, the enzymes do

not recognize a consensus sequence or structural motif. Structural studies of the human isoform GalNAc-T12 reveal a unique mechanism of substrate recognition that incorporates catalytic and lectin domain binding to previously glycosylated sites. The structure supports a model where each isoform obeys a distinct set of rules for substrate recognition that are defined by a combination of interactions between its catalytic domain and/or lectin domain and preferred residues on a substrate and/or previously glycosylated sites on a substrate.

(15) Optimization of expression of O-GlcNAc cycling enzymes for Cryo-Electron Microscopy

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O-GlcNAcylation is a dynamic post-translational modification (PTM) on thousands of nuclear, cytoplasmic and mitochondrial proteins; including transcription factors, tumor suppressors, kinases, phosphatases and histone modifying proteins. This PTM targets serine and threonine residues and frequently results in a crosstalk with phosphorylation. Moreover, protein *O*-GlcNAcylation is sensitive to UDP-GlcNAc (uridine diphospho-N-acetylglucosamine) concentration, the sugar donor utilized by OGT. This nucleotide sugar is derived directly from the nutrient dependent hexosamine biosynthetic pathway (HBP) and its concentration is directly influenced by nutrient availability. *O*-GlcNAcylation modulates protein homeostasis by changing its activity, interaction with protein partners, localization and degradation. As a result, *O*-GlcNAcylation plays an essential role in many cell signaling pathways and deregulation of this PTM has been linked to insulin resistance, diabetes, cancer, lupus and neurodegerative diseases.

Many PTM are regulated by multiple or sequential enzyme action. Nevertheless, only two enzymes control *O*-GlcNAcylation. The addition of N-acetylglucosamine onto target proteins is controlled by O-GlcNAc transferase (OGT), while removal of this residue is catalyzed by O-GlcNAcase (OGA). However, the details of how these enzymes interact with and recognize protein substrates is largely unknown. For example, we still do not understand how those two enzymes can act on so many different substrates. The lack of a detailed structure has been a major barrier in investigating OGT and OGA molecular mechanisms. A better understanding of these enzymes will surely accelerate the design of inhibitors that can be used as cellular probes or as potential therapeutic target. Although domains of the *O*-GlcNAc cycling enzymes have been solved by X-ray crystallography, the structure of the intact proteins remain unknown. The latest generation of cryo-Electron Microscopy (cryo-EM) equipment using direct electron detectors and software for automated collection of images, in combination with the use of advanced image-analysis methods, has dramatically increased resolution. Here, for the first time, we report the 2D classification of full length OGA (103 kDa) and OGT (110 kDa) by cryo-EM. We also show an OGA 3D model of compiled catalytic and stalk domain (7.5 Å). While we are still solving the 3D structure of the full length OGA and OGT, our preliminary observations suggest that the structures diverge from models built from the single crystalographically solved domains.

(16) Cell lineage impacts CD16a / Fc gamma Receptor 3a structure and antibody binding affinity through N-glycan composition

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CD16a/Fc gamma receptor IIIa activates a protective cytotoxic response following engagement with antibody clustered on the surface of a pathogen or diseased tissue. Therapeutic monoclonal antibodies (mAbs) with greater Fc-mediated affinity for CD16a on natural killer (NK) cells and monocytes show superior therapeutic outcome; however, one significant factor that promotes antibody-CD16a interactions, the asparagine-linked carbohydrates (N-glycans), remains undefined. Analysis of CD16a binding affinity in vitro revealed that two of the five possible N-glycans, at Asn45 and Asn162 contributed to antibody binding. We determined that CD16a from primary human NK cells contained a high percentage of hybrid-type N-glycans at Asn45 and the glycans at position Asn162 included complex-type biantennary, hybrids and oligomannose forms, depending upon the donor. Primary human monocytes expressed CD16a with predominantly oligomannose-type N-glycans at Asn45 and complex-type biantennary N-glycans at Asn162. Surprisingly, CD16a N-glycan composition affected antibody binding affinity. Recombinant CD16a with oligomannose N-glycans bound IgG1 Fc with 10 to 50-fold greater affinity than did CD16a having primarily complex-type and highly branched N-glycans. Furthermore, the sensitivity to N-glycan composition was attenuated in the CD16a N162Q variant but not the CD16a N45Q or N38Q/N74Q/N169Q variants. The changes in binding activity mirrored changes in NMR spectra of the two CD16a glycoforms, indicating that CD16a glycan composition also affects structure. Thus, CD16a from primary human NK cells and monocytes is compositionally, and likely also functionally, distinct. This study provides critical evidence that cell lineage determines CD16a N-glycan composition and antibody-binding affinity.

(17) Data in the driver's seat for exploring the glycome

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Glycosylation of proteins, lipids and proteoglycans in human cells involves at least 167 identified glycosyltransferases

(GTfs), and these orchestrate the biosynthesis ofdiverse types of glycoconjugates and glycan structures, resulting in a vast glycome. Pinpointingthe biological systems in which glycans play rolesis challenging, so we have recently been working on systems to shine light on the dark corners of the glycome via a genetic entry point. Mutations in the glycosyltransferase genome cause more than 58 rare, monogenic congenital disorders of glycosylation (CDGs). They are also statistically associated with a large number of complex phenotypes, diseases or predispositions to complex diseases based on Genome-Wide Association Studies (GWAS). CDGs are extremely rare and often with severe medical consequences. In contrast, GWAS are likely to identify more common genetic variations and generally involve less severe and distinct traits. We recently confirmed that structural defects in GTf genes are extremely rare, which seemed at odds with the large number of GWAS pointing to GTf-genes. To resolve this issue, we surveyed the GTf-genome for reported CDGs and GWAS candidates; we found little overlap between the two groups of genes. Moreover, GTf-genes implicated by CDG or GWAS appear to constitute different classes with respect to their: (i) predicted roles in glycosylation pathways; (ii) potential for partial redundancy by closely homologous genes; and (iii) transcriptional regulation as evaluated by RNAseq data. Our analysis suggest that more complex traits are caused by dysregulation rather than structural deficiency of GTfs, which suggests that some glycosylation reactions may be predicted to be under tight regulation for fine-tuning of important biological functions.

(19) Novel Insights into the Role of Glycans in the Bone marrow Niche

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Circulating platelet count is tightly regulated to avoid spontaneous bleeding or adverse thrombotic events. The role of lectin and glycans in thrombopoiesis and the bone marrow niche remains understudied. Sialic acid addition by sialyltransferases (STs) is often the terminal glycan modification. Heritable mutations in a gene essential for sialic acid biosynthesis, GNE, causes congenital thrombocytopenia. Platelet desialylation emerges as a potential diagnostic marker and therapeutic target for patients with immune thrombocytopenia (ITP). Genetic knockout of specific STs (St3gal1 and St3gal4) in mice also result in significant thrombocytopenia. St3gal4 adds sialic acids preferentially to N-linked glycans, St3gal1 acts specifically on O-linked glycans, demonstrating the distinct functions of STs.

Recent data showed that platelet desialylation induced by in vivo aging, sepsis and due to loss of St3gal4 lead to thrombocytopenia due to rapid clearance by the hepatic Ashwell Morell Receptor (AMR). Binding of the AMR by desialylated platelets stimulate hepatic thrombopoietin production, thus indirectly affecting the bone marrow niche. Thrombocytopenia is also observed in mice that lack St3gal1. Deletion of St3gal1 results in exposure of the tumor-associated Thomsen-Friedenreich (TF) antigen on O-linked glycans. Rather than causing accelerated platelet clearance, TF antigen exposure in megakaryocytes lead to defective thrombopoiesis induced by bone marrow resident immune cells. This presentation will discuss the contrasting effects of different sialic acid loss on platelet homeostasis, highlight novel glycan dependent pathways for platelet disorders, and discuss new therapeutic targets for platelet disorders.

(20) Site-1 protease is essential for activation of ER stress response for skeletal development but indispensible for the for the mannose-6-phosphate modification of lysosomal enzymes

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Site-1 protease (S1P), encoded by membrane-bound transcription factor peptidase, site 1 (*MBTPS1*), is a ubiquitously expressed serine protease localized in the Golgi apparatus. S1P proteolytically activate unique membrane-bound latent transcription factors such as sterol regulatory element binding proteins and activating transcription factor 6 in mice and in cultured cells. Recently, *in vitro* studies show that S1P activates N-acetyl glucosamine-1-phosphotransferase (GPT) by cleaving its α/β subunit precursor, which is required for the mannose-6-phosphate (M6P) modification of lysosomal enzymes in the Golgi apparatus for their targeted transport to the lysosome through specific M6P receptors. Defective M6P modification causes the lysosomal storage diseases mucolipidosis-II/III due to accumulation of un-digested macromolecules in lysosomes. However, how S1P differentially regulates these diverse functions in humans has been unclear. In addition, no human disease with S1P deficiency has been identified. Here, we report a pediatric patient with an amorphic and a severely hypomorphic mutations in *MBTPS1*. The unique compound heterozygotic mutation results in ~ 1% functional *MBTPS1* transcripts, which is associated with skeletal dysplasia and elevated blood lysosomal enzymes. We found that the residually expressed S1P is sufficient for lipid

homeostasis, but not for ER and lysosomal functions especially in patient chondrocytes. The defective S1P function specifically impairs activation of the ER stress transducer BBF2H7, leading to ER retention of collagen in chondrocytes. S1P deficiency also causes abnormal secretion of lysosomal enzymes. Collectively, these abnormalities lead to apoptosis of chondrocytes and lysosomal enzyme-mediated degradation of the bone matrix. Correction of an *MBTPS1* variant using antisense morpholino oligo or reduction of ER stress using small compound mitigated collagen trafficking defects. Interestingly, S1P-KO cells had less severe lysosomal phenotypes than in GPT-KO cells, indicating that S1P is an important but not the sole enzyme to proteolytically activate GPT. These results define a new congenital human skeletal disorder, and, more importantly, reveal S1P is particularly required for skeletal development in humans.

(21) Glycosyltransferase ST6Gal-I promotes pancreatic ductal adenocarcinoma progression and metastasis

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Sialyltransferase ST6Gal-I adds α2-6 sialic acids to select *N*-glycosylated cell surface receptors, thereby modulating receptor function and intracellular signaling. ST6Gal-I is upregulated in multiple human cancers, including >70% of pancreatic ductal adenocarcinoma (PDAC) samples. ST6Gal-I confers cancer stem cell (CSC) properties evidenced by tumorspheroid growth, chemoresistance and tumor initiating potential. As an example, we previously reported that ST6Gal-I imparts gemcitabine resistance by dampening DNA damage. One possible mechanism by which ST6Gal-I promotes a CSC phenotype is by increasing the expression of the master stem cell transcription factor, Sox-9. Forced overexpression or knockdown of ST6Gal-I directly regulates Sox9 expression in a wide array of established cancer lines. Sox9 is known to be a potent driver of acinar to ductal metaplasia (ADM), a key PDAC initiating event. To interrogate the role of ST6Gal-I and Sox9 in ADM, ST6Gal-I was overexpressed in the 266-6 acinar cell line. ST6Gal-I overexpression was found to increase expression of Sox9 and other ductal markers even in the absence of known ADM inducers TNFα and TGFβ. Contrarily, ST6Gal-I knockdown (KD) reduced expression of ductal markers and upon treatment with TNFα and TGFβ enhanced expression of acinar markers. Together, these results suggest that ADM depends on ST6Gal-I activity. Post ADM, PDAC progresses through the formation of PanINs. This process of progressive lesion formation leading to frank PDAC can be replicated in murine models of pancreatic cancer utilizing pancreas specific oncogenic K-Ras (KC). To investigate the relationship of ST6Gal-I and PDAC *in vivo*, we generated ST6Gal-I knock-in (KI) mice with oncogenic K-Ras (KC-ST6Gal-I KI). The KC-ST6Gal-I KI mice presented with significantly decreased overall survival (median = 4.3 months) compared to the KC mice (median = 14 months). This was coupled with a marked increase in liver and lung metastasis in KC-ST6Gal-I KI mice compared to KC mice. Given ST6Gal-I's role in driving CSC phenotype and upregulating Sox9, we hypothesized that the observed poor prognosis in our murine models might be due to early onset of ADM as well as enhanced CSC characteristic, which is a known cause of metastasis. To investigate this, we generated resident stem cell derived organoids from KC, KC-ST6Gal-I KI, ST6Gal-I KI and wild type (WT) mice. Compared to WT, ST6Gal-I KI organoids had increased cell proliferation. In the presence of oncogenic K-Ras, ST6Gal-I KI further enhanced proliferation compared to organoids with oncogenic K-Ras alone. Furthermore, KD of ST6Gal-I in KC organoids significantly reduced cell proliferation. In ongoing studies we are defining the signaling mechanisms underpinning a ST6Gal-I/Sox9 axis in PDAC initiation events and other aspects of CSC behavior. These collective studies highlight a novel role for tumor glycans in PDAC pathogenesis, and implicate ST6Gal-I as a promising therapeutic target.

(22) GOLPH3 promotes oncogenesis by controlling the intra-Golgi trafficking of glycosphingolipid synthases

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The *GOLPH3* oncogene is located on a human chromosome region, which is frequently amplified in solid tumours. *GOLPH3* encodes a Golgi complex associated protein involved in membrane trafficking and Golgi structure maintenance. While its

role in the stimulation of cell proliferation pathways is widely accepted, how GOLPH3 gain of function mediates oncogenesis is not completely understood. Here we show that GOLPH3 regulates proliferation by promoting sphingolipid glycosylation. Specifically, we found that GOLPH3 interacts with sphingolipid glycosylating enzymes at the Golgi complex and controls their intra-Golgi recycling during cisternal progression. Through this mechanism GOLPH3 counteracts sphingolipid glycosylating enzymes leakage to lysosomes and consequent degradation resulting in increased enzymes levels. Alterations in glycosylating enzymes proteostasis and sphingolipid metabolism induced by increased GOLPH3 levels impact on growth signalling pathways, thus promoting uncontrolled proliferation. Importantly, GOLPH3 overexpression/ amplification tightly correlates with tumour cells sensitivity to pharmacological inhibition of sphingolipid glycosylation suggesting that inhibition of sphingolipid metabolism represents a valuable therapeutic option for patients bearing GOLPH3 dependent tumours.

(23) A Role for Galectin Proteins and Glycans in Parkinson's Disease Pathogenesis

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Parkinson's disease is the second most common neurodegenerative disease, affecting approximately 1 million Americans and 10 million people worldwide. Misfolded accumulations of the protein alpha-synuclein is the pathological hallmark of disease and thought to underlie disease pathology. Specifically, amyloid forms ofα-syn can propagate between cells, leading to a spreading, progressive pathology in affected tissues and cause inflammation in these regions. We have investigated the mechanisms by which amyloid forms ofa-syn can enter naïve cells using an imaging based assay that relies on the recruitment of galectin proteins to ruptured vesicles. These studies reveal that amyloid forms ofa-syn, and other amyloid proteins associated with neurodegenerative diseases, can induce the permeabilization of vesicles following endocytosis. We observe that the inability to degrade these amyloids leads to progressive vesicular fusion, forming large proteinaceous inclusions in cells surrounded by a corona of galectin proteins, which we also observe in post-mortem brain samples from PD patients. We also observe thata-syn can be secreted from an affected cell in the context of extracellular vesicles positive for Gal3 and Gal8. Lectin based interrogation of these vesicles reveals that they possess a distinct glycan signature, compared to other extracellular vesicles. These findings highlight the role of galectin proteins in the pathogenesis of neurodegenerative diseases and suggest that specific glycans may facilitate the non-classical secretion ofa-syn in the context of extracellular vesicles.

(24) Turning Glycomics Into Lego-Toying

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The structural diversity of human glycans – the glycome – is vast and poses challenges for analytic and functional studies. The human genome contains over 200 distinct genes encoding glycosyltransferases, and our knowledge of the properties of these and their roles in the known glycosylation pathways in cells is relatively advanced. Currently some 170 glycosyltransferase genes can be assigned to rather specific roles in biosynthetic steps for the human glycome, although for most of the isoenzyme families our understanding of the unique functions of individual enzymes is still limited. Arguably though, current knowledge of assembly of the glycome makes it simpler to explore structure and functions of the glycome by a genetic entry point rather than from a structural entry point. With introduction of the facile nuclease-based gene-editing tools for knockout as well as site-directed knockin of glycosyltransferase genes the genetic approach to glycomics is becoming a standard tool in labs. Our group has widely applied the genetic approach for discovery and dissection of biological functions of protein glycosylation, and explored the engineering and design options for recombinant production of glycoprotein therapeutics and display of glycans. An overview of these efforts will be presented.

(25) Engineering cells via chemoenzymatic glycan editing

Peng Wu
The Scripps Research Institute

Complementary to metabolic oligosaccharide engineering, chemoenzymatic glycan editing has emerged as a powerful tool to modify glycan structures within a cellular environment. Using this approach, we can add novel functionalities to the cell surface to endow cells with new functions. In this talk, I will discuss our recent discovery of using fucosyltransferase and sialyltransferase to engineer immune cells for applications to cancer immunotherapy.

(26) Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery

Matthew P. DeLisa
Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University

The emerging discipline of bacterial glycoengineering has made it possible to produce designer glycans and glycoconjugates for use as vaccines and therapeutics. Unfortunately, cell-based production of homogeneous glycoproteins remains a significant

challenge due to cell viability constraints and the inability to control glycosylation components at precise ratios *in vivo*. To address these challenges, we describe a novel cell-free glycoprotein synthesis (CFGpS) technology that seamlessly integrates protein biosynthesis with asparagine-linked protein glycosylation. This technology leverages a glyco-optimized *Escherichia coli* strain to source cell extracts that are selectively enriched with glycosylation components, including oligosaccharyltransferases (OSTs) and lipid-linked oligosaccharides (LLOs). The resulting extracts enable a one-pot reaction scheme for efficient and site-specific glycosylation of target proteins. The CFGpS platform is highly modular, allowing the use of multiple distinct OSTs and structurally diverse LLOs. As such, we anticipate CFGpS will facilitate fundamental understanding in glycoscience and make possible applications in on-demand biomanufacturing of glycoproteins.

(27) Microbial glycoenzymes for carbohydrate synthesis and analysis

Sabine Flitsch
The University of Manchester

Carbohydrate active enzymes provide useful tools for the analysis and synthesis of glycans, but the toolbox of these biocatalysts is still limited in terms of reaction diversity and substrate range. We have initiated a programme to discover enzymes from microbial sources, some of which have been selected as homologues of mammalian enzymes. This lecture will discuss high throughput screening for established and new activities and discuss examples of substrate and reaction promiscuity that can be exploited by reaction engineering.

(28) Engineering the next generation of glycoprotein therapeutics and vaccines in bacteria

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Glycoprotein engineering in eukaryotic cell lines is constrained by the complex architecture of glycan biosynthesis pathways and their dependence on some types of glycosylation for survival. In our glycoengineering approach, we provide biosynthetic access to completely new glycoprotein structures by installing artificial N-glycosylation pathways in the cytoplasm of the biotechnological workhorse, *Escherichia coli*. These pathways rely on cytoplasmic N-glycosyltransferases (e.g. from *Actinobacillus pleuropneumoniae*), which install a single β-linked glucose onto asparagine residues in the Asn-X-Ser/Thr sequon. Recombinant proteins can be targeted for site-specific glycosylation by introduction of this short sequence motif, and the N-linked glucose serves as an efficient primer for synthesis of oligo- and polysaccharides directly onto proteins in the bacterial cytoplasm. We demonstrate biosynthetic pathways for modifying therapeutic proteins with a half-life extending polysaccharide, and the synthesis of vaccine candidates consisting of pathogen associated glycans presented on antigenic carrier proteins.

(29) New Platforms for the Discovery of Natural Siglec Ligands

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Sialic acid is abundant on mammalian cells and is used as a ligand by Siglecs, which are sialic acid-binding immunoglobulin-type lectins. Siglec-ligand interactions contribute to immune cell regulation in a variety of ways, but primarily as a means of inhibiting immune cell activation. There is a growing link between dysregulation of Siglecs and diseased states - such as cancer, neurodegeneration, and autoimmunity – motivating the need to better understand the sialic acid-containing glycans recognized by Siglecs. One of the challenges in studying Siglec-ligand interactions is that they are typically low affinity in nature. Avidity effects on a cell surface, and in particular within an immunological synapse, enables these weak interactions to manifest as biological effects. To advance our knowledge of Siglec ligands, our lab has begun developing new tools and approaches to better study these weak interactions that play contributing role(s) in health and disease. As Siglecs are cell surface receptors, a soluble version of Siglecs, expressed as Fc-chimeric proteins, have been instrumental in studying Siglec ligands. We have re-engineered such constructs to greatly increase the applications for which they can be used. These new constructs are being used in innovative ways to study Siglecs ligands, such as: mass spectrometry-based assays that can quantitatively measure interactions even in the mM range, flow cytometry analysis of Siglec ligands on immune cells, and incorporation of Siglecs onto platforms that present Siglecs in a highly multivalent manner. Progress will be presented on the ability of these new constructs to enable a deeper understanding of the glycan ligands of Siglecs in health and disease.

(30) Lighting up the rumen: using fluorescent glycan conjugates to visualize diverse metabolic capabilities of rumen microbiota at the single cell level

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Safe and sustainable beef production is a priority to the Canadian beef industry. As such, there is growing interest to

develop practices that maximize production outputs while mitigating environmental and economical costs. The rumen of beef cattle houses a diverse community of microorganisms that represents the most efficient bioreactor known in nature. The composition of this community impacts feed digestion, nutrient accessibility, host health, and waste production. Fibrolytic enzymes, beneficial live microorganisms (probiotics), and host-indigestible glycans (prebiotics) are promising candidates as next-generation feed additives to improve cattle growth performance. Current methods to investigate the relationships between beneficial bacteria and feed glycans in ecosystems such as the rumen are indirect and lack the high-resolution power necessary to identify and quantify interactions in complex communities. Rumen bacteria possess functionally diverse pathways to dismantle and metabolise glycans, which limits our ability to correlate metabolic phenotypes with genotypes. To address these limitations, we have developed first-in-class molecular tools to directly visualize polysaccharide uptake by rumen bacterial isolates using fluorescent glycan conjugates (FGCs) at the single-cell level. Yeast α-mannan (YM), a polysaccharide extracted from the cell wall of *Saccharomyces cerevisiae* and a common constituent of alternative feedstocks and prebiotics, was conjugated to a fluorescent probe (FLA-YM). FLA-YM was fed to pure bacterial cultures of *Bacteroides thetaiotaomicron (B.theta)*, a well-studied intestinal symbiont with YM metabolic potential, a *B.theta* mutant with no potential to utilize YM, and strains of bacteria isolated from the rumen and enriched on YM. Uptake of FGCs presented here provides direct evidence of genotypes endowed with YM metabolic potential, which enables rapid identification of YM utilization. In support of visualized interactions, we have performed a series of indirect methods, including growth kinetics, whole genome sequencing, and RNA-Seq to demonstrate variations in YM-utilization profiles of rumen *Bacteroides* spp. at the strain level.

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

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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.

We aim to make this method a widespread tool for both, glycoproteomics experts and non-experts and have begun the transfer of the method to interested laboratories by supplying them with a novel approach to generate meaningful glycoproteomic datasets. We also plan an evaluation of the IsoTag workflow via interlaboratory comparison of identical samples. This small round-robin will be the basis for standardized procedures for an IsoTag 'kit'. These will then be tested for general applicability by dissemination - along with a set of standards - to a larger group of laboratories (mainly MS core facilities). After evaluation these results will be available in an online repository (www.IsoStamp.org).

We also demonstrate the versatility of the IsoTag method through use in additional systems and are currently in the process of developing new IsoTag probe systems for new or specialized applications. We are highly interested in starting collaborations to test and improve our IsoTag technology and are able to provide the IsoTag probes, standards and protocols to interested laboratories.

(32) Capsular glycolipids in Gram-negative bacterial pathogens

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University of Guelph

Capsules are surface polysaccharide structures that protect bacteria from phagocytosis and/or complement-mediated killing. They are essential virulence determinants for many pathogens. Many different repeat-unit structures have been determined for capsular polysaccharides (CPSs) isolated from a wide range of bacteria. Despite this diversity, biochemical and bioinformatics data reveal a conserved assembly strategy that is used for capsule production by important Gram-negative human pathogens including *Bordetella pertussis*, *Campylobacter jejuni*, extraintestinal pathogenic *Escherichia coli* (ExPEC), *Haemophilus influenzae*, and *Neisseria meningitidis*. The same approach is thought to be used by high-profile respiratory pathogens of livestock such as *Bordetella bronchiseptica*, *Mannheimia haemolytica*, and *Pasteurella multocida*. The assembly pathway has been elucidated primarily in ExPEC and various aspects have been validated experimentally in other bacteria. These CPSs are assembled on a conserved reducing terminal glycolipid acceptor composed of phosphatidylglycerol modified with ~5-10 residues of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Two CMP-Kdo-dependent glycosyltransferase enzymes assemble the β-Kdooligosaccharide on the terminal lipid. KpsS adds a single residue and KpsC possesses two catalytic sites with different linkage specificities to generate an oligosaccharide product with alternating β-2,4 and β-2,7-Kdo residues. Solved structures of thecatalytic domains of KpsC indicate it isderived from a GT-B fold but dramatic reduction in the N-terminal Rossmann-like

domain results in a structure resembling a modified GT-A fold. Because the β-Kdo oligosaccharide is essential for capsule production and elimination of the capsule structure attenuates survivalof these pathogens in appropriate animal models, these enzymes offer potential targets for small-molecule inhibitors. To that end, a high-throughput format assay has been developed using a single catalytic domain of KpsC and an initial screen of a 140,000-compound library has been completed.

(33) An in vitro gut-immune model illuminates N-glycosylation-dependent host-pathogen interactions of Campylobacter jejuni

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NCTC 11168 *Campylobacter jejuni* pathogenicity was studied in a human immune-competent intestinal model,through the lens of its N-linked protein glycosylation (Pgl) pathway. The ability of this Gram-negative enteropathogen to infect and colonize avian and mammalian model organisms has been directly linked to the Pgl pathway, but the exact role of *C. jejuni* N-glycans in causingdisease in humans is unclear. To address this, an accessible*in vitro* gut-immune model more closely resembling a human intestinal microenvironment was employed to characterize several changes in *C. jejuni* epithelial invasion, immunogenicity, and virulence factors. Glycosylation-deficient Δ*pglE*, lacking the N-linked heptasaccharide modification of its proteome, was 100-fold less capable of adhering to and invading this intestinal model in cell infectivity assays. Chemokine and cytokine quantification by immunoassay revealed that Δ*pglE* differentially modulated inflammatory responses in different intestinal microenvironments, suppressive in some but activating in others. Virulence-associated outer membrane vesicles produced by wildtype and Δ*pglE C. jejuni* were shown to have differential composition and function by activity-based protein profiling analysis, with wildtype vesicles able to rescue Δ*pglE* infectivity to wildtype levels in infection experiments. Overall, use of the *in vitro* gut model allowed for further characterization of the multifaceted importance of the Pgl pathway in *C. jejuni* host-pathogen interactions in human contexts. We anticipate these methods will be broadly applicable to further studies of *C. jejuni* and to other enteropathogens of interest.

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(34) Immunological characterization of protein glycosylation in Streptococcus pneumoniae

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*Streptococcus pneumoniae (*Spn) is still a major human pathogen causing a variety of invasive pneumococcal diseases (IPD) with alarming mortality rates. Spn can be divided into over 90 serotypes based on the composition and structural differences of their capsular polysaccharides (CPSs). Since the introduction of the 7-valent (PCV7) and 13-valent (PCV13) glycoconjugate vaccines, effective against the most prevalent serotypes of Spn, the incidence rates of IPD in children have been reduced significantly. However, a global serotype distribution shift has highlighted the importance of generating improved pneumococcal vaccines to include a wider range of serotypes. Moreover, increasing numbers of clinical isolates from IPD patients are nonencapsulated Spn (NESpn), indicating the emergence of pathogenic NESpn strains. The serotype distribution shift and increasing NESpn in the clinical isolates necessitate the urgent investigation of serotype-independent, conserved, protective subunit vaccine targets. Spn surface proteins are major virulence factors and conserved immunogens and, therefore, Spn proteins have been examined as vaccine candidates, without considering potential post-translational modifications. Current knowledge on the potential glycosylation of Spn surface proteins, such as Pneumococcal serine-rich Protein (PsrP), and how this modification impacts virulence and immunogenicity is surprisingly very limited. We hypothesize that protein glycosylation is essential for Spn virulence and carbohydrate epitopes from surface glycoproteins of Spn can induce protective adaptive immune responses. Here, we characterize Spn protein glycosylation and identify glycoproteins as major targets for vaccine design. We have collected evidence for the glycosylation of highly immunogenic, conserved, pneumococcal surface proteins, and characterized pneumococcal glycosyltransferases that are instrumental in protein glycosylation, and contribute to Spn virulence.

(36) The use of glycoengineered cell lines for investigating influenza A interactions with modified sialic acids

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Influenza A viruses (IAV) are an important human pathogen causing 3 to 5 million cases of severe illness and 290,000 to 650,000 deaths globally each year (WHO, 2018). IAVs have broad host ranges and are able to infect a wide array of animal species including humans, pigs, horses, dogs, waterfowl, and domestic poultry. IAV use sialic acids (Sia) as the primary receptor for infection via the hemagglutinin (HA) and

neuraminidase (NA) glycoproteins. Sia are found in large amounts both on the cell surface as part of the glycocalyx and in mucus that protects the respiratory and GI tracts. Sia may be chemically modified (including 7,9-O-, 9-O-acetyl, 5-N-glycolyl) and are attached to glycan chains through different linkages, which vary between hosts and tissues. While the importance of Sia α2,3- and α2,6-linkages to IAV tropism and evolution have been well studied, the roles of modified Sia in IAV host adaptation are not well known. Modified Sia have been identified as inhibitors of NA and HA, but their role during infection is unclear. We have shown previously that 7,9-O- and 9-O-Ac Sia are expressed in many IAV host tissues and are highly expressed in the respiratory tissue of humans, particularly in the submucosal glands. 7,9-O-, and 9-O-acetyl modifications are also expressed in embryonated chicken eggs used to grow vaccine strains, and on MDCK, HEK293, and A549 cells used in IAV research. 5-N-glycolyl modifications (Neu5Gc) are highly expressed in some tissues in natural IAV hosts like pigs and horses, and in IAV model species such as mice and guinea pigs, but are not found in humans, chickens, and ferrets. To understand the effects of these modified Sia on IAV tropism, we have developed glyco-engineered cell lines that either lack or over-express 7,9-O- and 9-O-Ac or Neu5Gc. MDCK, HEK293, and A549 cells were engineered using CRISPR/Cas9 and expression plasmids, and were used to determine the impact of modified Sia on IAV enzyme function, virus growth, and host adaptation. Preliminary results show that 7,9-O- and 9-O-Ac modifications decrease NA cleavage and HA binding in a strain specific manner. The presence of 7,9-O- and 9-O-Ac Sia in respiratory mucus may be a functional point of contact between virus and these Sia modifications during infection *in vivo*. Through the over-expression of CMAH, we can generate human and canine cells displaying Neu5Gc to levels observed in natural host tissues. This provides an *in vitro* platform to assess the effect of Neu5Gc modification on HA and NA function, and potential adaptation in serial passage. We also observed that 7,9-O- and 9-O-Ac modifications are highly variable in expression, showing incomplete population homogeneity and modifications as a minority of total Sia on the surface of cells, and we are therefore also examining the regulation of their expression on cells.

(37) Targeting STT3A-Oligosaccharyltransferase Causes Herpes Simplex Virus 1 Dysfunction

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Herpes simplex virus 1 (HSV-1) is a contagious neurotropic enveloped herpesvirus responsible for oral cold sores, other mucous membrane lesions, and herpesviral encephalitis. The HSV-1 envelope contains N-glycosylated proteins involved in viral attachment and spread, and are therefore candidate drug targets. NGI-1 inhibits the oligosaccharyltransferase (OST) complexes STT3A-OST and STT3B-OST, which catalyze cotranslational and posttranslational N-glycosylation, respectively. Since host OSTs attach glycans to asparaginyl residues of both host and viral polypeptides, they are essential for HSV-1 envelope biogenesis.

Here, we evaluated HSV-1 N-glycosylation and infectivity using NGI-1 and the NGI-1 analogue C19 in conjunction with knockout lines for OST isoform-specific subunits. HSV-1 infection and N-glycosylation of two envelope proteins (gC and gD) were primarily dependent upon STT3A-OST, but STT3B-OST could partially replace STT3A-OST. In cells lacking STT3B-OST function due to knockout of either the catalytic subunit STT3B or the oxidoreductase subunits MagT1/TUSC3, and thus entirely dependent upon STT3A-OST for N-glycosylation, NGI-1 robustly suppressed production of plaque-forming units (a measure of infectivity) but not the unglycosylated capsid protein VP5 (a measure of viral particles). Consequently, pharmacological targeting of STT3A-OST in the absence of STT3B-OST resulted in dysfunctional HSV-1 particles in otherwise nearly normal numbers.

Counterintuitively, HSV-1 infections of different NGI-1 treated OST knockout lines revealed that HSV-1 dysfunction did not correlate with total envelope protein hypoglycosylation. NGI-1 caused similar degrees of dysfunction with HSV-1 from either infected wild type cells or cells unable to cotranslationally N-glycosylate envelope proteins (due to knockout of either the catalytic subunit STT3A or the translocon-docking subunit DC2 of STT3A-OST). However, NGI-1 caused more hypoglycosylation of gC and gD in STT3A-OST-impaired cells than wild type cells. Cells lacking STT3B-OST activity had an opposite relationship, with NGI-1 being relatively more effective for causing HSV-1 dysfunction than hypoglycosylation.

Taken together, efficacy of OST inhibitors toward HSV-1 infection appears to depend upon blocking attachment of a key subset of envelope N-glycans that are necessary for viral particle function, but not particle number, and are normally added cotranslationally by STT3A-OST but can be alternatively attached posttranslationally by STT3B-OST. This suggests that an effective anti-HSV-1 strategy might require inhibition of both OST isoforms, resulting in attenuated infectivity while yielding defective viral particles with envelope proteins lacking immunosuppressive "glycan shields".

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(38) African trypanosomes evade immune clearance by O-glycosylation of the VSG surface coat

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The African trypanosome Trypanosoma brucei spp. is a paradigm for antigenic variation, the orchestrated alteration

of cell surface molecules to evade host immunity. The parasite elicits robust antibody-mediated immune responses to its variant surface glycoprotein (VSG) coat, but evades immune clearance by repeatedly accessing a large genetic VSG repertoire and 'switching' to antigenically distinct VSGs. This persistent immune evasion has been ascribed exclusively to amino-acid variance on the VSG surface presented by a conserved underlying protein architecture. However this model does not account for the scope of VSG structural and biochemical diversity. Recent structural and biochemical work demonstrates that certain VSGs (with VSG3 as the prototypical member) contain an O-linked carbohydrate at the top of the coat-exposed surface. Mass spectrometric analyses indicate that this O-glycosylation site is heterogeneously occupied both in VSG3 (by zero to three hexose residues) and in other VSGs. As well, this O-glycosylation increases parasite virulence by impairing the generation of protective immunity. These data alter the paradigm of antigenic variation by the African trypanosome, expanding VSG variability beyond amino-acid sequence to include surface post-translational modifications with immunomodulatory impact.

(39) O-fucosylation of nucleocytosolic proteins in Toxoplasma gondii and Cryptosporidium parvum

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Toxoplasma gondii and *Cryptosporidium parvum* are two medically relevant parasites. *T. gondii* infection can lead to developmental defects in fetuses and neurological damages in immunocompromised individuals, while *C. parvum* causes severe diarrhea and has been identified as one of the leading causes of diarrhea-related deaths in infants in developing countries.

We have previously shown that *T. gondii* modifies with *O*-fucose Ser/Thr residues on at least 70 proteins involved in mRNA processing, nuclear transport (including nucleoporins), and signaling. Structured illumination microscopy, using the fucose-specific *Aleuria aurantia* lectin (AAL), localizes the *O*-fucosylated proteins to assemblies at the nuclear periphery, in close proximity to nuclear pore complexes.

T. gondii encodes for an orthologue of SPINDLY (*Tg*SPY), the recently identified plant nuclear *O*-fucosyltransferase (OFT) and a paralog of *O*-GlcNAc transferases (OGT). *Tg*SPY localizes to both cytosol and nucleus of the parasite and its knockout causes loss of AAL binding and a deficit in parasite growth. Additionally recombinantly expressed *Tg*SPY can hydrolyze GDP-fucose, when tested against a panel of GDP- and UDP-sugars, and can fucosylates itself *in vitro*. Taken together these results strongly support *Tg*SPY as the parasite OFT. At a molecular level, in Δ*spy* mutants we observe a decrease in the abundance and/or stability of proteins that are fucosylated in parent cells, *e.g.* Nup68YFP. Additionally, in about 10% of the Δ*spy* population, both a tagged version of endogenous *Tg*GPN (*O*-fucosylated in wild type) and a YFP reporter with a nuclear localization signal (not a target of *Tg*SPY) 'leak' from the nucleocytosol into the parasitophorous vacuole.

AAL also binds to the nuclear periphery of *C. parvum* sporozoites and lectin-enrichment followed by LC-MS/MS analysis identifies two putative *C. parvum* nucleoporins as *O*-fucosylated. Like *T. gondii*, *C. parvum* encodes for a SPINDLY orthologue. However, previously published work biochemically characterized *Cp*SPY as an OGT and the enzyme was unable to restore AAL-binding to *T. gondii* Δ*spy*. We have recombinantly expressed *Cp*SPY to further characterize its sugar nucleotide donor specificity. Furthermore, as no conserved *de novo* or salvage pathway for GDP-Fuc biosynthesis is apparent in *C. parvum*, we will verify the presence of GDP-Fuc in the parasite sugar nucleotides pool and search for biosynthetic or import pathways responsible for its synthesis or uptake.

These studies will lead to a better understanding of the biochemistry of SPY enzymes in both parasites and will also provide insights on nuclear glycosylation in eukaryotes.

(40) Capsule synthesis in the fungal pathogen *Cryptococcus neoformans*

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Cryptococcus neoformans is an opportunistic pathogen that causes serious infections in hundreds of thousands of people each year, primarily in developing areas of the world. Cryptococcosis is contracted by inhalation of the infectious particle, which grows in the lungs; depending on the host immune status the infection may be cleared, remain latent in the lungs, or disseminate. Dissemination to the central nervous system results in a potentially lethal meningoencephalitis, with mortality rates in some areas close to 70%. Current therapy for this disease is inadequate.

Like *S. cerevisiae, C. neoformans* produces a predominantly carbohydrate cell wall and also glycosylates proteins and lipids, although its repertoire of sugar moieties is broader than that of the model yeast. In addition, *C. neoformans* displays a large polysaccharide capsule outside of its cell wall; capsule polysaccharides are also shed copiously into the environment. The capsule, which is unique among fungal pathogens, is required for virulence. Capsule thickness (and probably capsule structure as well) is extremely sensitive to environmental conditions.

This presentation will consider aspects of both capsule synthesis and capsule regulation. We have recently described several nucleotide sugar transporters that supply precursors for capsule synthetic reactions. The absence of these transporters significantly perturbs cryptococcal pathogenesis and also alters the host response to this pathogen. We are also working to define capsule regulation at the transcriptional level and to relate the transcriptional network to the

underlying glycobiology of capsule synthesis, particularly the production and transport of nucleotide sugar donors.

(43) Glycan glucuronylation balances cell signaling during ovarian development

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Three of the major O-linked glycans in Drosophila are either branched or capped with glucuronic acid (GlcA). One of these O-linked structures is of special interest because it is built on an O-linked Fuc core, suggesting that it may contribute to Notch function. In order to better understand the potential functions of glycan glucuronylation in Drosophila, we screened candidate glucuronyltransferase (GlcAT) genes for mutations that affect glucuronylation of O-linked glycans. An insertional mutant in one of these candidates, GlcAT-S, showed reduced glucuronlyation of both core 1 disaccharide and O-Fuc structures. We generated a new GlcAT-S mutant by mobilization of the P-element. In the resulting P-excision line, GlcAT-Scms, homozygous females failed to lay eggs. Analysis of O-linked glycans in GlcAT-Scms ovaries revealed a significant decrease in glucuronylated core 1 disaccharide. Further analysis of the GlcAT-Scms infertility phenotype demonstrated that egg development arrests around stage 2 in association with altered architecture of the egg chamber and over-proliferation of follicle cells, a phenotype previously linked to altered Notch signaling. Immunofluorescence analysis of Notch in GlcAT-Scms ovaries revealed Notch expression is significantly higher and is upregulated in less mature ovarioles in the mutant than in wildtype. Notch and hedgehog pathways operate sequentially during ovariole development to initiate formation of the egg chamber, drive oocycte development, and induce endoreplication in follicle cells at the appropriate time. Ovarioles in GlcAT-Scms mutant ovaries have increased hedgehog expression, fail to undergo oocyte differentiation, and follicle cells do not enter into endoreplication despite the associated overexpression of Notch and Hindsight. These phenotypes are consistent with mutations in the Hedgehog pathway that cause the Hedgehog pathway to be continuously active and indicate the importance of proper glycan glucuronylation for critical signaling pathways that regulate Drosophila ovary development.

(44) Glycosphingolipid Metabolic Reprogramming Drives Neural Differentiation

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Neural development is accomplished by differentiation events leading to metabolic reprogramming. Glycosphingolipid metabolism is reprogrammed during neural development with a switch from globo to ganglio-series glycosphingolipids production. Failure to execute this glycosphingolipid switch leads to neurodevelopmental disorders in humans, indicating that glycosphingolipids are key players in this process. Nevertheless, both the molecular mechanisms that control the glycosphingolipid switch and its function in neurodevelopment are poorly understood. Here, we describe a self-contained circuit that controls glycosphingolipid reprogramming and neural differentiation. We find that globo-series glycosphingolipids repress the epigenetic regulator of neuronal genes expression AUTS2. AUTS2 in turn, binds and activates the promoter of the first and rate limiting ganglioside producing enzyme GM3 synthase, thus fostering the synthesis of gangliosides. By this mechanism the globo-AUTS2 axis controls glycosphingolipid reprogramming and neural genes expression during neural differentiation, which involves this circuit in neurodevelopment and its defects in neuropathology.

(45) Significant roles of O-glucose glycans in mammalian Notch trafficking and signaling

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It has been ten years since *Rumi*, protein *O*-glucosyltransferase 1 (*POGLUT1*), was discovered as an essential component for Notch signaling in *Drosophila*. What have we learned from *Rumi*? The Notch signaling pathway is an evolutionarily well-conserved, intercellular signaling pathway required for proper cell-fate decision. Mammalian Notch receptors utilize a large extracellular domain that consists of up to 36 epidermal growth factor-like (EGF) repeats for ligand binding. EGF repeats are modified with three different types of *O*-glycans; *O*-glucose, *O*-fucose, and *O*-GlcNAc. These modifications are required for full Notch activity. POGLUT1 adds *O*-glucose to a serine residue within a consensus sequence $C^1XSX(P/A)C^2$ of EGF repeats. *O*-Glucose can be extended to a linear trisaccharide, Xylα1-3Xylα1-3Glc, by the sequential actions of two glucoside xylosyltransferases (*GXYLT1/2*) and xyloside xylosyltransferase 1 (*XXYLT1*). Addition of *O*-fucose or *O*-GlcNAc is catalyzed by protein *O*-fucosyltransferase 1 (*POFUT1*) or EGF domain-specific *O*-GlcNAc-transferase (*EOGT*), respectively. Mutations in *POGLUT1*, *POFUT1*, and *EOGT* have been found that are linked to human diseases including muscular dystrophy. The crystal

structures of POGLUT1, XXYLT1 and POFUT1 in complex with substrates provided the structural basis for *O*-glycosylation on EGF repeats. These findings clearly indicated the importance of *O*-glycosylation in regulation of Notch receptor function, but not the underlying molecular mechanism. We recently demonstrated that endoplasmic reticulum (ER)-localized POGLUT1 and POFUT1 can distinguish between folded and unfolded EGF repeats. This discovery, together with our previous finding that POFUT2 plays a role in folding of thrombospondin type 1 repeats, has led us to propose a non-canonical, likely general, ER quality control mechanism for the folding of small cysteine-rich motifs. Consistent with the hypothesis, we show here that cell-surface expression of endogenous NOTCH1 in HEK293T cells depends on the presence of *POGLUT1* and *POFUT1* in an additive manner. *In vitro* unfolding assays reveal that addition of *O*-glucose or *O*-fucose stabilizes a single EGF repeat, and that addition of both *O*-glucose and *O*-fucose enhances stability, also in an additive manner. Furthermore, we solved the crystal structure of a single EGF repeat modified with an *O*-glucose trisaccharide at 2.2 Å resolution. The structure reveals that the linear glycan bends back to fill up an extended surface groove of the EGF with multiple contacts with the protein, providing a chemical basis for the stabilizing effects of the glycans. Collectively, this work suggests that *O*-fucose and *O*-glucose glycans cooperatively stabilize individual EGF repeats through intramolecular interactions, thereby regulating Notch trafficking in cells. Currently, we are investigating the effect of xylosyl-extension of *O*-glucose glycans on Notch function. Supported by NIH grant GM061126 (to R.S.H.) and KAKENHI 17H06743 (to H.T.).

(47) Protein O-mannosylation regulates axon wiring in the nervous system of Drosophila

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Protein O-mannosylation (POM) is mediated by several glycosyltransferase enzymes that attach O-linked mannose to serine and threonine residues of secretory pathway proteins. POM is highly conserved in evolution, with orthologues of POM-generating enzymes being present in animal organisms from *Drosophila* to humans. These enzymes show distinct specificities towards protein substrates. Protein O-mannosyltransferases 1 and 2 (POMT1/2) are known to modify Dystroglycan (Dg) and promote its ligand-binding activity essential for muscle function. Mutations affecting biosynthesis of O-mannosyl glycans on Dg result in severe congenital muscular dystrophies. *POMT* mutations also affect neural development, but the function of POM in the nervous system is poorly understood. With remarkable conservation of POMT functions, *Drosophila* offers a useful model to investigate the role of POM in neural development. *Drosophila POMTs* were found to regulate sensory feedback required for patterning of peristaltic muscle contractions that control body posture. In *POMT* mutant embryos, sensory neurons properly send axons to the CNS, but their connectivity inside the CNS is abnormal. Mutant sensory axons show defects in branching and crossing midline of the ventral ganglion, which results in thickened commissural branches and depleted longitudinal tracts. Normal wiring of axon termini can be restored in *POMT* mutants by transgenic expression of POMTs in sensory neurons, which suggests that *POMTs* have sensory neuron-specific functions. Genetic analyses indicated that POM targets besides Dg could regulate wiring of sensory axons. Involvement of POMTs in control of coordinated muscle contractions and proper connectivity of sensory axon termini uncovers novel POM functions that may be conserved in humans and can potentially shed light on neurological pathomechanisms of dystroglycanopathies.

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(48) Roles of EGF repeat xylosylation in mammalian development and Notch signaling

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Glucoside xylosyltransferases-1 and -2 (GXYLT1 and GXYLT2) add the first xylose to O-glucose-modified serine residues in epidermal growth factor-like (EGF) repeats with a specific consensus sequence. This is followed by the function of xyloside xylosyltransferase 1 (XXYLT1), which extends the xylose-glucose-O disaccharide by adding a second xylose to it. Our published and preliminary data in flies indicate that Drosophila Notch signaling is highly sensitive to the level of xylose residues on the Notch receptor (Lee et al. PLoS Genetics, 2013; Lee, Pandey and Jafar-Nejad, PLoS Genetics, 2017). However, the role of EGF repeat xylosylation in mammalian Notch signaling and Notch-independent processes has yet to be elucidated. Human XXYLT1 is frequently amplified in specific types of cancer in which Notch signaling has a tumor suppressor function. Lower expression levels of Gxylt2 mRNA due to aberrant DNA methylation in patients with ulcerative colitis has been reported, although to date, to our knowledge, there is no report that links GXYLT1 to any human diseases. In this study, we sought to determine the consequences of disrupting the function of individual xylosyltransferases in a mammalian context. We generated loss-of-function alleles of all three mouse xylosyltransferases using CRISPR-Cas9 technology on a C57BL/6 background. From sibling crosses between heterozygous animals for each mutant, we have obtained a number of Gxylt2–/– and Xxylt1–/– pups but no Gxylt1–/– pups. Further analyses showed that the observed lethality in Gxylt1 knockout animals occurs around mid-

gestation, suggesting an early and crucial developmental role for this enzyme. We are in the process of isolating mouse embryonic fibroblasts (MEFS) from these animals as well. I will report the results of our ongoing research on EGF repeat xylosylation by using the above-mentioned animals and cellular models.

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(49) Neonatal ST6Gal-1 Expression Alters Microbiome Composition and Mucosal Immunity

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The symbiosis between the host and its microbiome is an ongoing interaction influenced by dietary patterns, environmental exposure, and host genetics. Commensal bacteria can advantage the host through production of digestible nutrients and education of mucosal immune cells. However, it is unclear whether the host can alter bacterial colonization to safeguard these benefits. The sialyltransferase ST6Gal-1 constructs a2,6-linked sialic acid onto the termini of N-linked glycans. Although ST6Gal-1 has an well-described role in epithelial cell stemness and immune cell development, its role in mucosal tissues during early development remains elusive. Early studies documented the loss of a2,6-linked sialic acids upon weaning in the newborn gastrointestinal tracts of rats and humans, but the biologic importance of this phenomenon has never been understood. We show that, in mouse models, ST6Gal-1 is expressed in small and large intestinal enterocytes in the neonatal period, resulting in a wholesale but temporary change in the sialylation of the intestinal epithelium. Largely driven by the hepatocyte-associated P1 promoter, this expression is absent by 24 days, concurrent with a reduction in blood ST6Gal-1 to steady-state adult levels. We hypothesized that this mucosal expression of ST6Gal-1 facilitates the critical early-life colonization of specific bacterial species by providing sialylated glycoproteins for adhesion or metabolic consumption. 16 S rRNA sequencing of fecal pellets indicated that, by 20 days of age, although total bacterial diversity was unchanged, striking differences in microbiome composition have manifested at the class, family, and genus levels. By comparison of altered original taxonomic units (OTUs) between ST6Gal-1 expressing and deficient mice on two genetic backgrounds, a list of bacterial genera highly dependent on host ST6Gal-1 expression was generated. We observed a reduction in Coprobacillus, Coprococcus, Adlercreutzia, and Prevotella, and an increase in Bilophila and Helicobacter in ST6Gal-1 deficient mice. Several of these genera have been previously reported to be either metabolically dependent on sialic acid or susceptible to sialic acid toxicity. Interestingly, these changes were also associated with a decreased polarization of lamina propria CD4+ T cells into Treg and Th1 subsets in St6gal1-KO mice. Our results suggest developmentally-regulated expression of an endogenous glycan-modifying enzyme can alter bacterial colonization and T cell polarization in early life.

(50) Structural and biochemical insights into the mechanism of plant polysaccharide acetylation

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Glycans are the most abundant biopolymers in Nature, forming the cell walls encasing every plant cell. Understanding enzymes involved in plant polysaccharide biosynthesis at the molecular level is paramount for gaining fundamental insight into how these biocatalysts work together to build architecturally complex structures such as plant cell walls. Xylan O-acetyltransferases catalyze the addition of O-acetyl moieties onto xylan, which are the predominant substituents on the third most abundant biopolymer on earth. We used a multipronged approach involving mammalian cell culture expression, X-ray crystallography, mutagenesis and molecular simulations to evaluate plausible substrate binding modes and provide detailed insights into the mechanism of the enzyme family involved in plant polysaccharide O-acetylation. (This research was supported by The Center for Bioenergy Innovation (CBI), a U.S. Department of Energy Research Center supported by the Office of Biological and Environmental Research. We alsothank the Center for Plant and Microbial Complex Carbohydrates (DESC0015662) for equipment support.)

(51) Strength through diversity: The evolution of plant cell wall glucan-active enzymes in GH16

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The evolution of specialized cell walls in land plants is one of the key features that enabled adaptation to diverse terrestrial environments. Plant cell walls are complex composite materials, which are primarily comprised of polysaccharides, *viz.* cellulose, hemicelluloses, and pectins. Plant morphogenesis therefore requires a large complement of carbohydrate-active enzymes for assembling, remodeling, and recycling cell wall components. The *xyloglucan endo-transglycosylase/hydrolase (XTH)* gene family in plants encodes enzymes of central importance to cell wall remodeling [1]. However, the evolutionary history of plant *XTH* gene products is incompletely understood *vis-à-vis* the larger body of Glycoside Hydrolase Family 16 (GH16) members, including their

closest sister group, the bacterial mixed-linkage endo-beta(1-3)/beta(1-4)-glucanases (MLGases).

We have recently combined molecular phylogeny, enzymology, and structural biology to reveal an ancient clade of dual-specificity MLGases/xyloglucanases from plants ["endo-glucanase 16" (EG16) members] that represent an extant, transitional group in the evolution of GH16 [2]. To gain broader insights into the distribution and frequency of EG16 and other GH16 members in plants, public genome and transcriptome databases were extensively mined to build a comprehensive census among 1300 species spanning early eukaryotes, algae, and recent plant lineages [3]. Crystallography of an exemplar *Vitis vinifera* (grape) EG16 in complexes with cognate substrates provided structural insight into the evolutionary trajectory of plant EG16 members within GH16, which will inform future genomic and functional studies across species [4]. Recent results toward ascertaining the biological role of EG16 enzymes in plants will be discussed.

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(52) Sphingolipid glycosylation and its role in membrane organization and plant-microbe interactions

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Glycosylinositol phosphorylceramides (GIPCs) represent the major sphingolipids in plants and constitute 25-40% of the plasma membrane lipids. GIPCs are also present in fungi, whereas glycosphingolipids in animals are similar but have a different structure. GIPCs have been difficult to study compared to other lipids and surprisingly little is known about their structure and biological functions. We have now identified several glycosyltransferases directly involved in GIPC glycan biosynthesis and have obtained a number of mutants that have enabled better understanding of the importance of GIPCs. Here we report the discovery of a GIPC GlcNAc-transferase, GINT1. In Arabidopsis GINT1 is only expressed in seeds, while other tissues express GMT1, a GIPC mannosyl-transferase instead. Arabidopsis *gint1* mutants have subtle phenotypes showing a slightly improved germination under salt stress. The GINT enzyme is located in the Golgi and uses UDP-GlcNAc as substrate. We have identified a UDP-GlcNAc transporter, UGNT1, required for biosynthesis of GlcNAc-containing GIPCs. Loss-of-function mutants in *UGNT1* are also virtually unable to add GlcNAc to protein N-glycans in the Golgi and accumulate high mannose structures, similarly to *cgl1* mutants deficient in N-acetyl-glucosaminyl transferase I, GNT1. In *Medicago truncatula* roots the mannosylated GIPCs are most abundant, but the GlcN(Ac) GIPC forms are also present. Downregulation of *GINT1* strongly impairs the symbioses with nitrogen-fixing rhizobia in root nodules and with arbusular mycorrhizal fungi leading to the senescence of symbiotic membrane structures but does not affect root growth. These results indicate that symbiosis-induced reprogramming of GIPC glycosylation is critical for the development, function, and persistence of plant-perimicrobial membranes during nodulation and arbuscular mycorrhizal symbiosis. Arabidopsis *gmt1* mutants have severely reduced growth and accumulate salicylic acid. Expression of *GINT1* in the *gmt1* mutant background can only partially rescue the growth phenotype, further supporting the conclusion that the different GIPC forms have unique functions that we are only beginning to understand.

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(53) Functional characterization of strain-specific glycan metabolism and single-cell visualization of glycan uptake within bacteria from the rumen microbiome

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Carbohydrates that comprise the cell wall of plants and fungi represent underexploited opportunities to selectively modulate intestinal microbiomes with the goal of benefiting

animal production. Selective feed ingredients, such as chemically unique polysaccharides (e.g. yeast mannan), have the potential to stimulate dynamic changes in community structure and induce improvements in feed digestibility and efficiency, and host performance. Commonly, microbial responses to dietary polysaccharides are studied indirectly, primarily by sequencing methods, which has created a bottleneck in translating sequence information into function. In this regard, molecular tools and "direct" methods that help unravel the mechanisms driving polysaccharide-microbe interactions in higher-throughput will benefit how we interpret and manipulate functional changes in microbiome communities. To address this, our group has been developing a multifaceted approach, involving selective anaerobic isolation, comparative metabolic pathway and CAZome (i.e. entire collection of carbohydrate active enzyme genes encoded within a single genome) fingerprinting analyses, RNA-sequencing, single cell super resolution structured illumination microscopy of fluorescent carbohydrate uptake, and streamlined enzymology, to evaluate saccharolytic responses of intestinal microbiomes to dietary polysaccharides. In this talk, I will present high-resolution *in silico* analysis, X-ray crystallography, selective growth profiling, and single-cell fluorescence microscopy to demonstrate that related strains of rumen *Bacteroides* spp. have evolved tailored pathways for the competitive utilization of yeast mannan in the cattle rumen ecosystem.

(54) Impact of cell wall carbohydrates on plant growth

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The plant cell wall is at the interface of the protoplast with the external environment. It contains structural and matrix polysaccharides that are synthesized either at the plasma membrane or in the Golgi apparatus. The loss of cell wall carbohydrates, such as pectins, negatively affects plant growth. Intriguingly, an overabundance of matrix polysaccharides, such as mixed-linkage glucan (MLG), also leads to a growth disruption. However, increasing the levels of pectin methylesterification has a beneficial impact on plant biomass accumulation. Therefore, plant growth depends on the homeostasis of cell wall polysaccharide levels and composition. Modifications of the cell wall in efforts to improve plants as sustainable resource of feedstock for bioenergy applications need to be tailored to overcome such a potential bottleneck. In this presentation, we will discuss our recent work on the study of pectin and MLG biosynthesis as well as relocalization of the biosynthetic machinery within the cell. Our results contribute a better understand the topology of plant cell wall polysaccharide synthesis and provide foundations to overcome growth penalty due to cell wall alterations in efforts to improve plants as bioenergy feedstock.

(55) A Unique Hydroxyproline-rich Glycoprotein from the green microalga Botryococcus braunii

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The green microalgal *Botryococcus braunii* grows in colonies of cells held together by a complex extracellular matrix (ECM) and is mostly studied for the liquid hydrocarbons it produces, which are stored in the ECM and can be converted into combustion engine fuels. The ECM of *B. braunii* is made up of cross-linked hydrocarbons, a carbohydrate retaining wall, a 2-3 μm long polysaccharide fibrillar sheath radiating outward from the retaining wall, and a single protein. The polysaccharide fibers accumulate to high levels in the growth media and maybe useful for several industrial applications. While the *B. braunii* ECM cross-linked hydrocarbon network has been well studied, the polysaccharide and protein portion of the ECM has been under studied. In recent years we have studied the single ECM protein and its relationship to polysaccharide fiber formation. Dividing *B. braunii* cells shed off the apical portion of the ECM, termed "shells", containing the polysaccharide fibers, retaining wall, and the single protein. Glycosyl composition analysis indicates the polysaccharide fibers mainly consist of galactose followed by arabinose and a deoxyhexose with minor amounts of glucose. The ECM protein, termed polysaccharide associated protein (PSAP), was identified by peptide mass fingerprinting and bioinformatics, contains several hydroxyproline (Hyp)-rich regions, and staining studies indicate PSAP is a glycoprotein. Mass spectrometry analysis identified ten *N*-linked PSAP glycosylation sites composed of sevendifferent mannose and *N*-acetylglucosamine containing glycans. Additionally, three of these glycans contain fucose and one of them is unusual since it is further modified with arabinose. Hyp residues in hydroxyproline-rich glycoproteins are often *O*-glycosylated and we found four short *O*-linked arabinose and galactose containing glycans on four consecutive Hyp, one of which also contains a 6-deoxyhexose. Finally, PSAP localization to the surface of the retaining wall where the polysaccharide fibers are attached was confirmed by fluorescence microscopy suggesting a role for PSAP in fiber synthesis. The identification of these unique glycans further expands the understanding of the unique metabolism carried out by this alga and offers isights into potential applications of *B. braunii* metabolic products.

(56) GAUTs synthesize diverse pectic HG glycans in structurally and functionally distinct plant cell wall polymers

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Homogalacturonan (HG) is a cell wall pectic glycan that contributes to plant cell wall structure, plant growth, cell adhesion and development. HG is synthesized by the *galacturonosyltransferase* (*GAUT*) gene family. We show that at least 6 of the 15 Arabidopsis GAUTs encode HG-biosynthetic galacturonosyltransferases (GalATs) and propose that different GAUTs synthesize HG in unique polymers with different functions in the wall. In support, different *gaut* mutants have diverse and severe growth phenotypes, such as *gaut1* mutants which are severely stunted. Interestingly, while total cell wall GalA content of *gaut1* mutant knockout suspension culture cells does not differ from WT cells, detailed characterization of the HG affected shows that GalA content is markedly reduced in cell wall fractions most tightly bound into the wall. These results lead to the hypothesis that GAUT1 synthesizes a specific pectin HG domain tightly integrated into the wall and crucial for wall integrity. In contrast, GAUT4 synthesizes the bulk of HG in poplar and switchgrass and its knockdown expression results in loss of the majority of the pectic polysaccharide, rhamnogalacturonan II, leading to the hypothesis that GAUT4-synthesized HG resides in independent HG and/or HG-RGII wall polymers.

We previously showed that GAUT1 and GAUT7 form a heteromeric enzyme complex in *Arabidopsis thaliana*. To explore the molecular functions of GAUT in this complex, we co-expressed GAUT1 and GAUT7 in HEK293 cells and produced a soluble GAUT1:GAUT7 complex that catalyzes elongation of HG products *in vitro*. Reaction rates, progress curves, and product distributions exhibited major differences dependent upon the degree of polymerization (DP) of the oligosaccharide acceptor. GAUT1:GAUT7 synthesized high-molecular-weight polymeric HG (> 100 kDa) in a substrate concentration–dependent manner typical of distributive (non-processive) glycosyltransferases with DP11 acceptors. Reactions primed with short-chain acceptors resulted in a bimodal product distribution of glycan products. Although, such bimodal product distributions have previously been used as evidence for processive elongation by glycosyltransferases, our data better support a two-phase elongation model in which a slow phase is distinguished from a phase of rapid elongation of intermediateand long-chain acceptors. The GAUT1:GAUT7 complex provides an example of a distributive glycosyltransferase that catalyzes polymerization of high-molecular-weight polysaccharides and has full activity only if incubated with acceptors longer than a critical chain length. (Supported by USDA AFRI 2010-65115-20396 and the BioEnergy Science Center and Bioenergy Innovation which are US Department of Energy Bioenergy Research Centers supported by the Office of Biological and Environmental Research in the Department of Energy's Office of Science. Also partially funded by Department of Energy Center Grant DESC0015662.)

(57) Targeting Oncofetal Chondroitin Sulfate for Cancer Therapy and Diagnosis

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Cancer is the biggest global health problem affecting the modern world. Developing therapeutics targeting cancer is extremely difficult due to the self-nature and the immense heterogeneity between different cancers in terms of type, origin, and underlying oncogenic mechanism. This has lead to the hunt for cancer specific targets common to all cancer types. We have described a common oncofetal Chondroitin Sulfate (ofCS) signature shared by placental and almost all cancer cells. In this we have shown that the recombinant *plasmodium falciparum* malarial VAR2CSA (rVAR2) protein, which has been evolutionarily selected for placental ofCS adherence, binds nearly all cancer cells and cancer tissues with almost no reactivity towards normal tissue. We have further shown that ofCS is a potential target for cancer specific therapy and diagnosis strategies. CS is involved in almost all the hallmarks of cancer through its placement on proteoglycans in the cell membrane and in the extracellular matrix. In line with this we have shown that blocking ofCS function in cancer cells of different origin using rVAR2 inhibits integrin related signaling, adhesion to ECM components, cellular detachment and cellular migration and invasion. Collectively these data demonstrate the existence of a specific and functional carbohydrate modification present only on cancer cells, and notably on almost all cancer cells and tissues. This suggests that ofCS has the potential to have a major impact on cancer diagnosis, monitoring of cancer progression, and cancer therapy.

(59) New Frontiers in Rotavirus and Human Milk Oligosaccharide Interactions

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Interaction with cell surface glycans is an essential and critical step in the pathogenesis of many enteric infections. Our work focusses on glycan interactions of rotavirus, the leading cause of diarrheal mortality in children < 5 years of age particularly in low- and middle-income countries. In the last 5 years, we and others discovered that several human rotavirus strains bind non-sialylated glycoconjugates present in mucosal secretions. This finding was paradigm shifting in our understanding of the infectivity of human rotaviruses.

Many new studies demonstrate that genetically-regulated differences in histo blood group antigen (HBGA) glycan expression affect susceptibility to diarrhea with different rotavirus strains and possibly, susceptibility to live, attenuated rotavirus vaccines. Since structural analogs to cell surface glycans are present in breast milk, we asked the question: How do maternal breast milk oligosaccharides affect rotavirus infectivity, particularly in neonates? Using a multidisciplinary approach involving virologists, structural biologists, glycobiologists, physicians, and epidemiologists, we are testing the hypothesis that complex interactions between intestinal glycan expression during neonatal development, human milk oligosaccharides (HMO) and breast milk microbiome affect clinical outcome of neonatal rotavirus infections. These studies are primarily carried out with a bovine-human reassortant rotavirus strain that almost exclusively infects neonates. In addition, analysis of glycan interactions of globally dominant rotavirus strains illustrates clear viral-genotype-dependent differences in glycan specificity, with implications for host specificity, zoonotic transmission and age-restriction of human rotaviruses. Recently, we have begun utilizing a new model of the human gastrointestinal epithelium to answer fundamental questions on how glycan expression affect rotavirus infection. Advances in the field of stem cell biology have facilitated the development of non-transformed, human intestinal epithelial cultures called human intestinal enteroids (HIEs) that recapitulate many biological and physiological properties of the human small intestine. We are using HIEs to understand the molecular basis of virus-glycan interactions. Our present studies serve as a foundation for future work on understanding the effect of breast milk components on clinically relevant rotavirus strains and vaccines, as well as other enteric pathogens where glycans are critical for infection.

(60) Targeting pathogenic polyglucosan bodies in Lafora disease using an antibody-enzyme fusion

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Lafora disease (LD) is a fatal childhood epilepsy and a non-classical glycogen storage disorder with no effective therapy or cure. LD is caused by recessive mutations in the *EPM2A* or *EPM2B* genes that encode the glycogen phosphatase laforin and an E3 ubiquitin ligase malin, respectively. A hallmark of LD is the intracellular accumulation of abnormal and insoluble α- linked polysaccharide deposits known as Lafora bodies (LBs) in several tissues, including most regions of the brain. These deposits contain an abnormal form of glycogen known as polyglucosan characterized by increased chain lengths and elevated phosphate. In mouse models of LD, genetic reduction of glycogen synthesis eliminates LB formation and rescues the neurological phenotype. Since multiple groups have confirmed that neurodegeneration and epilepsy result from the accumulation of this aberrant polysaccharide, a major focus in the field has shifted toward the development of therapies that reduce glycogen synthesis or target LBs for degradation with the goal of treating LD.

LBs have previously been considered to be "diastase-resistant." Herein, we identify the optimal enzyme for degrading LBs is α-amylase. We developed a therapeutic agent by fusing human pancreatic α-amylase to a cell-penetrating antibody fragment to form an antibody-enzyme fusion (AEF) called VAL-0417. We designed a novel protocol for purifying native LBs from *Epm2a-/-* and *Epm2b-/-* mice which we characterize by light and electron microscopy. We show that VAL-0417 robustly degrades LBs *in vitro*, and we define the soluble oligosaccharides released by VAL-0417 by HPAEC-PAD. VAL-0417 shows robust cellular uptake, degrades glycogen and LBs *in situ*, and significantly reduces the LB load *in vivo* in both muscle and brain of *Epm2a-/-* mice. VAL-0417 is a promising therapeutic for the treatment of LD and a putative precision therapy for an intractable epilepsy. AEFs represent a new class of antibody-based drugs that could be utilized to treat glycogen storage disorders and other diseases.

(61) Glycoengineering using GlycoCRISPR toolbox-Towards Cell-based Glycan Array for Dissection of Interactions with Glycans

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Glycan arrays have played a pivotal role in exploring the informational content of complex glycans. Current glycan arrays display oligosaccharides that are generated by chemical and chemoenzymatic synthesis or isolated from various biological sources. In common to these arrays are that they are limited resources, produced through considerable efforts, and they display individual saccharides without the natural

context of other glycans and glycoconjugates at the cell surface. The structural diversity of complex glycans on human cells – the glycome – is vast, and despite advancements in analytic strategies it continues to be difficult to decipher the biological roles of glycans with respect to specific glycan structures, type of glycoconjugate, particular glycoproteins, and distinct glycosites on proteins. In contrast to this, the number of glycosyltransferase genes involved in the biosynthesis of the human glycome is manageable, and the biosynthetic roles of most of these enzymes are defined or can be predicted with reasonable confidence. Thus, with the availability of the facile CRISPR/Cas9 gene editing tool it now seems easier to approach investigation of the functions of the glycome through genetic dissection of biosynthetic pathways, rather than by direct glycan analysis. Here, we present a self-renewable cell-based glycan array that differentially displays the most important glycan features of the human glycome via a large library of genetically engineered isogenic HEK293 cells using GlycoCRISPR, a library of validated high-efficiency gRNA designs targeting of the human glycosyltransferase. The broad utility of this cell-based glycan array is demonstrated by dissecting glycan-binding specificities of lectins, Abs and other glycan binding proteins.

(62) Targeting sialoglycan-mediated immune suppression for cancer immunotherapy

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Escape of tumor cells from immune control is a hallmark of cancer. This immune escape is facilitated by the generation of an immune-suppressive tumor microenvironment. Various mechanisms have been implicated in the generation of cancer-associated immune suppression, such as the upregulation of inhibitory receptors on cells of the adaptive immune system including CD8 T cells, loss of antigenicity of tumor cells and the influx of immune suppressive leukocytes into the tumor microenvironment. Recent approaches by targeting inhibitory pathways in T cells such as the PD-1/PD-L1 axis or CTLA-4-mediated inhibition have led to exciting responses in various cancer and have been prove of concept that the cancer-associated immune suppression can be reversed. However, only a minority of patients responds to the currently available immunotherapies. Thus, new strategies to reverse cancer-associated immune suppression are urgently needed.

In cancer, the microenvironment is significantly changed compared to normal tissue. In particular, there is a general increase of sialic acid-containing glycans that are recognized as self-associated molecular patterns. Such 'hypersialylation' in cancer can enhance interactions with sialic acid binding lectins including Siglecs, which are able to significantly influence anti-tumor immunity and cancer progression. Engagement of immune-suppressive Siglecs - expressed on various immune cell types - can mediate immune evasion of tumor cells. Our recent analyses of tumor-infiltrating leukocytes from patients with non-small cell lung cancer, colorectal cancer and epithelial ovarian cancer have revealed the presence of inhibitory CD33-related Siglecs (CD33rSiglecs) including Siglec-9 on myeloid cells, T cells and NK cells.

The presence of inhibitory Siglecs has been shown by others and our group to significantly suppress anti-tumor immune reactions by innate and adaptive immune cells in*in vitro*cellular systems and*in vivo*mouse models. It is therefore conceivable to develop agents that target this Siglec/sialoglycan axis in cancer to further improve anti-cancer immunity and reverse cancer-associated immune suppression. We therefore developed*in vitro*platforms to screen for inhibitors of sialoglycan-mediated interactions and genetic mouse models that allow testing of potential inhibitors*in vivo*. Importantly, our murine tumor models are syngeneic wherein the adaptive immune response can be fully investigated and combination immunotherapies with PD-1 or CTLA-4 can be studied.

(63) Thermodynamic Profiling of Binding of Human Macrophage Galactose-Type Lectin and Synthetic Tn-Bearing MUC1 Glycopeptides Analogs

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Mucin 1 (MUC1) *O*-glycosylation, is commonly truncated in malignant and premalignant epithelia. The human C-type lectin receptor, macrophage galactose-type lectin (MGL), expressed by both dendritic cells and macrophages, has been identified as a receptor of Tn and sTn antigens (CD175/175a). We here introduce Isothermal Titration Calorimetry (ITC) to study the thermodynamics of the interaction. Our approach combined synthesis of a structurally well-defined MUC1 glycopeptide models. A panel of *O*-glycosylated peptides with the MUC1 tandem repeat sequence, HGVTSAPDTRPAPGSTAPPA, was prepared. Presence of ordered structures was detected by circular dichroism (CD) spectroscopy. Stabilization by formation of a polyproline II (PP II) stretch was observed with increasing glycan epitope density. ITC data analysis revealed the enhancement of affinities of MGL for multivalent MUC1 glycopeptide ligands. The measured dissociation constants (K_d) ranged from 6 μM for mono- to 600 nM for triple-glycosylated MUC1 peptide, respectively. All studied interactions, regardless of the glycan's site of attachment or density, exhibited enthalpy-driven thermodynamics. To probe into the role of the solvent we studied thermodynamics in D_2O. Differences in enthalpy-entropy compensations were observed amongst tested glycopeptides.

(64) Impact of signal peptide mutations on secreted glycoproteins

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Maturation of secretory proteins is frequently initiated by signal peptide assisted translocation of the growing protein chain to the endoplasmic reticulum (ER) and Golgi compartments. Signal peptides are responsible for recognition and binding of nascent protein to the cytosolic signal recognition particle, its insertion into ER-conducting channels, and further protein maturation, including glycosylation. Non-synonymous genomic variants affecting sequence of signal peptides, especially its hydrophobic core, modify signal peptide function with consequences to the expression or processing of the mature secretory protein. Our in silico screen of genomic variants that affect signal peptides identified several hundred proteins predicted by SignalP4.1to have modified or lost signal peptide function. Although SP mutations haven't been studied extensively, single AA variants of several proteins were implicated in various human diseases, such as defective dentine biomineralization, familial isolated hypoparathyroidism, permanent neonatal diabetes, heritable connective tissue disease or rare coagulation factor VII deficiency. To study consequences of the single amino acid substitutions in single peptide, we have selected inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) protein, previously characterized in our lab, as a model system. We used bioinformatics approach to predict potential function modifying substitutions in its hydrophobic core and we selected two mutations predicted to cause loss of function phenotype. Wild type and both variant signal peptides were expressed in HEK293F cells under serum-free cell culture conditions. All of the clones expressed mRNA in similar amounts, but both mutant variants abolished secretion of mature ITIH4 protein. From the filtered genomic and somatic mutations predicted as a loss of SP function we selected 8 additional glycoproteins, and created over 30 mutants to study the impact of the SNVs and level of the correct predictions in model cell lines. The protein expression level and glycosylation were evaluated. We found that two SP mutants of LYZL2 and SULF1 prohibited secretion or protein production and one of the SHBG mutants expressed protein with longer N-terminal peptide.

(65) Defining O-GlcNAc Cycling Rates Utilizing Dynamic Isotopic Detection of Amino Sugars with Glutamine (IDAWG)

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The post-translational modification, O-linked β-N-acetylglucosamine (O-GlcNAc) is found on thousands of nuclear and cytosolic proteins in mammals and is thought to be a regulatory modification playing a role in a variety of cellular processes. A single gene encodes the enzyme O-GlcNAc-transferase (OGT) allowing for the addition of O-GlcNAc onto Serine and Threonine residues, while another gene encodes for O-GlcNAc-hydrolase (OGA), which facilitates the removal of the aforementioned modification. It is hypothesized that O-GlcNAc is a dynamic modification; that is, the modification exhibits a shorter half-life than the modified protein. Furthermore, it is believed that dynamics vary among different modification sites as well as the proteins that are modified and that levels, localization, and kinetics of the cycling influence this property. Unfortunately, dynamics has only been evaluated on a small number of O-GlcNAc modified proteins due to the laborious and insensitive methods utilized. Therefore, there is a need in the field to develop high-throughput, sensitive methods to evaluate the dynamics of O-GlcNAc and O-GlcNAc modified proteins.

To address this gap in knowledge, a novel methodology will first be established. The method known as Dynamic Isotopic Detection of Aminosugars With Glutamine (IDAWG) was originally designed as a quantification tool for glycomics to look at released glycans labeled with amide-^{15}N-Gln taking advantage of the hexosamine biosynthetic pathway. Here, we seek to develop a Dynamic IDAWG approach coupled with innovative enrichment techniques to look at intact O-GlcNAc modified glycopeptides. The culmination of these techniques should allow for examination of the dynamic nature of the O-GlcNAc modification and the modified proteins to be observed specifically in any cell line.

(66) Regulation of terminal N-glycan modifications by bisecting GlcNAc

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Bisecting GlcNAc, a central GlcNAc branch of N-glycan highly expressed in brain, is synthesized by GlcNAc transferase GnT-III. We previously found that GnT-III deficient mice showed improved Alzheimer's pathology with reduced amyloid plaques, indicating that bisecting GlcNAc promotes Alzheimer's disease pathology. However, the physical functions of bisecting GlcNAc remain unclear.

In the present study, we 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 of N-glycans with fucose and sialic acid are largely increased in the knockout brain. In addition, a major brain-type terminal epitope of N-glycan, HNK-1, was also upregulated. These results show that various terminal modifications of N-glycans are upregulated by the loss of bisecting GlcNAc. As to the mechanism, we revealed that most

of the biosynthetic enzymes for these glycan epitopes, including fucosyltransferases, sialyltransferases and HNK-1-related enzymes, commonly show poor activity toward N-glycan substrates with bisecting GlcNAc, as compared with non-bisected type counterparts. These findings suggest that bisecting GlcNAc serves as a biosynthetic inhibitor for various terminal modifications of N-glycans through enzymatic properties.

(67) Identification and characterization of UDP-mannose in human cell lines and mouse organs: Differential distribution across brain regions and organs

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Nucleotide sugars are donor substrates for glycosyltransferase and are thus vital regulators of glycosylation. Guanosine diphosphate mannose (GDP-Man) and dolichol phosphate-mannose serve as donor substrates for mannosylation and are used in N-glycosylation, O-mannosylation, C-mannosylation, and the synthesis of glycosylphosphatidylinositol-anchor. Yet, very few nucleotide sugars have been identified in mammals compared to other organisms. We hypothesized that mammals possess previously unidentified nucleotide sugars that regulate mammalian glycosylation.

Here, we identified low-abundant nucleotide sugars by using an improved LC-ESI-MS/MS method that we described previously (Nakajima et al. 2010, 2013). We for the first time detected low-abundant uridine diphosphate-mannose (UDP-Man), which can serve as potential donor substrate, exists in mammals. The cellular UDP-Man level was elevated in a mannose dose-dependent manner. Liquid chromatography-mass spectrometry analyses showed that mouse brain, especially hypothalamus and neocortex, contains higher concentrations of UDP-Man compared to other organs. These findings indicate that in mammals the minor nucleotide sugar UDP-Man regulates glycosylation, especially mannosylation in specific organs or conditions. Because only a limited number of nucleotide sugars are known to be involved in glycosylation, the discovery of UDP-Man in mammals will provide new insights into our understanding of novel regulatory mechanisms of glycosylation.

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(68) Fucose Metabolism: Rethinking old concepts and identifying new mechanisms

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The biochemical pathways for synthesizing GDP-Fucose have been known for many years. Elegant studies in HeLa cells from in 1975 showed that the*de novo*pathway, which converts GDP-Mannose to GDP-Fucose, is by far the major source (>90%), but a minor salvage pathway contributes <10%, starting from L-fucose.

We revisited this pathway in various cell types using differentially labeled C^{13}isotopes of glucose, mannose and fucose to better understand the contributions of both the*de novo* and salvage pathways to N-linked fucosylation. We show that when available, L-fucose not only becomes the preferential source of fucose in N-glycans, but it actually suppresses the contribution of the*de novo*pathway. This suppressive effect was specific for the production of GDP-Fucose from the*de novo*pathway, since it did not alter the contributions of glucose and mannose to GDP-Mannose in N-glycans. This strongly supports the idea that there are separate pools of GDP-Fucose.

Another mystery in fucose metabolism involves the protein responsible for transporting L-fucose into the cell. Previous studies have shown that L-fucose is transported by a glucose insensitive transporter, but that transport can be partially impeded when non-specifically blocking typical GLUT family members. We utilized the colorectal carcinoma line HCT116, which lacks the*de novo*pathway but retains the normal salvage pathway, to perform a siRNA screen for genes capable of blocking L-fucose dependent fucosylation.

Our siRNA screen consisted of approximately 150 transporters and identified Glut1as a candidate transporter whose knockdown most significantly affected savage pathway-dependent fucosylation. Importantly, we utilized two independent methods, siRNA knockdown and Glut1 specific inhibitors, to validate Glut1 as a bona fide candidate. Interestingly, both siRNA knockdown and chemical inhibition of Glut1 could be overcome in a dose dependent fashion with excess L-fucose, suggesting that another transporter is functional at a higher concentration of L-fucose.

Here we provide novel insights into how the fucose*de novo*and salvage pathways interact to insure fucosylation and how L-fucose is transported into mammalian cells.

(69) Structural insights into GalNAc-T4 preference for prior glycosylated substrates

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Mucin type O-glycosylation is initiated by a large family of polypeptide GalNAc transferases (GalNAc-Ts), with ~ 20 isoforms in mammals that transfer GalNAc onto specific Thr or Ser residues of substrate proteins. Our prior enzyme specificity studies have shown that different isoforms prefer Thr acceptors over Ser acceptors at different rates. Each

isoform also recognizes varying sequence motifs and remote prior glycosylation (O-GalNAc) in an N- or C-terminal direction. Interestingly, several isoforms (GalNAc-T4, T7, T10, T12) also recognize neighboring prior glycosylation. Our lab has shown that GalNAc-T4 recognizes both long range and short range N-terminal prior O-GalNAc glycosylation. Here we report, structural and kinetic studies on wildtype GalNAc-T4 and selected GalNAc binding mutants.

Structurally, nearly all GalNAc-T isoforms contain a catalytic domain and a lectin domain connected by a flexible linker. Here we have obtained an X–ray crystal structure of GalNAc-T4 isoform bound to a diglycopeptide substrate containing both long range and short range prior GalNAc glycosites. In this structure, we have for the first time identified the catalytic domain and likely lectin domain residues that interact with the short range prior glycosite. Interestingly, the catalytic domain GalNAc binding residues are close to aromatic residues that recognize the TPGP substrate sequence motif that is common to most isoforms. We have performed kinetic studies on an array of glycopeptide substrates with a fixed TPGP motif with varying long range and short range prior Thr-O-GalNAc glycosites relative to the acceptor site. Lectin and catalytic domain GalNAc binding mutants of GalNAc-T4 have also been studied against these substrates and give the expected changes in enzyme activity and catalytic efficiency. Importantly, we have shown that we can selectively eliminate the long range, short range or both prior glycosite preferences of GalNAc-T4. In addition, we have observed that the diglycopeptide susbtrate shows substrate inhibition kinetics which we believe is dependent on differential binding preferences between the long range and neighboring prior glycosites.

In summary, GalNAc-T4 prefers a diglycoeptide substrate (with both neighboring and remote O-GalNAc) better than a mono-glycopeptide (with either neighboring or remote O-GalNAc) and naked unglycosylated peptide substrates. Overall, this work reveals the mechanistic basis for both long range and short range glycosylation preferences of the GalNAc-Ts. This work also demonstrates that we can selectively eliminate the different prior glycosite preferences of a GalNAc-T.

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(70) Spatial organization of glycosylation enzymes in the Golgi determines its glycan output

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The Golgi apparatus is the organelle where majority of the cellular glycosylation reactions take place, to modify both proteins and lipids. The importance of this processing reaction in human physiology is highlighted by the numerous disorders that are associated with its impairment. While the glycans produced by a given cell type are ultimately determined genetically, how the cell *translates* this linear genetic information into a complex branched glycan structure is not clear. The mammalian Golgi apparatus is organized as series of stacked cisterna displaying a polarized structure with the cargoes entering on its cis-side and leaving on the trans-side. The glycosylation enzymes show a non-uniform distribution across cisternae with tendency for the early acting enzymes to be localized to the cis-side and the late acting ones to the trans-side. While such polarized localization of the enzymes has been proposed to have a functional significance in determining the glycan output of the Golgi, the issue has not been experimentally explored extensively. Here, by using glycosphingolipid (GSL) biosynthesis as a model system we show that polarized spatial organization of the GSL biosynthetic enzymes indeed determines the GSL output of the Golgi. Further we also show that there is dedicated molecular machinery to maintain this organization. In particular, Grasp55, a protein involved in the stacking of the Golgi cisternae, influences the proper intra-Golgi localization of a key GSL biosynthetic enzyme, lactosylceramide synthase, by regulating its entry into retrograde transport vesicles. Deletion of Grasp55 function leads to a mislocalization of this enzyme, which we show can change the GSL output of the Golgi. These findings underscore the importance of the Golgi organization in determining the glycan output and leads us to suggest that membrane transport processes *translate* the genetic information into a specific configuration of the Golgi, described in terms of the glycosylation enzyme localization, which then *determines* the glycan output of the Golgi.

(71) Human Milk Oligosaccharide Analysis of large sample cohorts using a novel Capillary Electrophoresis Platform

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Introduction: In previous studies we have presented GlyQ™, a cartridge-based capillary electrophoresis instrument that allows for the rapid analysis of fluorescently labeled

carbohydrates. In this study, we have used the Gly-Q™ platform for the analysis of human milk oligosaccharides (HMOs).

The reliability and validity of the Gly-Q platform for the analysis of HMOs was determined by the parallel comparison of Gly-Q results with standard analytical methods, in this case high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) and triple quadrupole mass spectrometry (QQQ). Initially ten separate individual human milk samples were compared and after validation a second sample set composed of human milk produced by nine individuals at four different time points (weeks 1, 2, 3 and 4 of lactation) was studied using this analytical platform.

Finally, to demonstrate the high-throughput capabilities of the Gly-Q™ instrument, two hundred fifty human milk samples from nineteen different milk banks all belonging to the Human Milk Bank Association of North America (HMBANA) were analyzed.

Methods: For Gly-Q analysis, human milk oligosaccharides were purified by centrifugation and ethanol precipitation, labeled by reductive amination with APTS and purified by solid phase extraction. For QQQ and HPAEC-PAD analysis, HMOs from each sample were isolated via chloroform methanol extraction, protein precipitation, and solid phase extraction using non-porous graphitized carbon cartridges.

Results: In the Gly-Q™ platform reliability study, all the paired samples studied yielded 100% concordance (in terms of secretor phenotype status identification) when compared with the other two methods.

For the lactation study (N = 29), it was found that the relative concentration of 2-FL/3-FL increases as lactation advances while the rest of HMOs decrease or stay constant. Non-secretor phenotype is characterized by the lack of LNFP-I independently of the lactation moment. Higher relative concentration of LNFP-II and LNDFH-II were found in non-secretor samples. These results are in agreement with previous studies.

From the two hundred fifty human milk samples analyzed from human milk banks, 82.4% (n = 206) contained secretor-specific oligosaccharides, lower than expected. We speculate that secretor-specific oligosaccharides may be degraded during acquisition, storage, or processing and prior to distribution. Relative oligosaccharide abundance observed in both secretor and non-secretor donor milk may be modified by HMBANA processing, thus these findings may not generalize to other donor milk entities.

(72) Improved Profiling of Sialylated N-Linked Glycans by HPAE-PAD

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Profiling a glycoprotein's asparagine-linked (*N*-linked) glycans is an important quality control assay for determining the fidelity and consistency of recombinant therapeutic glycoprotein production. It is also an important assay when evaluating changes in production conditions, comparing expression systems, and comparing a biosimilar to an innovator's product. Good profiling requires high resolution separation of a glycoprotein's released glycans. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established technique for this assay which is orthogonal to other techniques such as capillary electrophoresis and hydrophilic interaction liquid chromatography with fluorescence detection. HPAE-PAD is especially effective for separating sialylated glycans, but there is opportunity to improve resolution. We evaluated changes to commonly used HPAE-PAD conditions to improve resolution. HPAE-PAD separations of *N*-linked glycans typically use 100 mM sodium hydroxide with a gradient of sodium acetate at 30 °C. Using the *N*-linked glycans released from bovine fetuin, bovine thyroglobulin, bovine fibrinogen, and human alpha 1 acid glycoprotein, we evaluated the effects of temperature and sodium hydroxide concentration on resolution. We show that lowering temperature and increasing sodium hydroxide concentration improve resolution. We also evaluated column format changes to reduce analysis time as well as sample and reagent consumption. Using a short column we are able to quickly evaluate the charge status of a glycoprotein's *N*-linked glycans. This may be an effective way to quickly screen the impact of changes in cell culture conditions on sialylation.

(73) Exploring the significance of xylosyl-extension of O-glucose glycans on EGF repeats for Notch signaling in mammals

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Notch signaling is an evolutionarily conserved cell-to-cell communication pathway which is necessary for development and homeostasis. In the extracellular domain of mammalian Notch receptors, there are 29 to 36 epidermal growth factor-like (EGF) repeats which are modified with *O*-linked glycans such as *O*-fucose, *O*-glucose and *O*-GlcNAc. Among them, *O*-glucose monosaccharides are attached to multiple EGF repeats which contain a distinct consensus sequence, $C_1XSX(P/A)C_2$, and can be extended to a trisaccharide form: Xyl-α1,3-Xyl-α1,3-Glc-β1-*O*-Ser. Genetic and biochemical approaches have indicated that *O*-glucosylation by protein *O*-glucosyltransferase 1 (*POGLUT1*, *Rumi* in *Drosophila*) is essential for Notch signaling in mice and *Drosophila*, while xylosyl-extension by a glucoside xylosyltransferase, *Shams*, negatively regulates Notch signaling in *Drosophila*. Recently, our knockout study using HEK293T cells showed that the absence of *POGLUT1* decreased the cell-surface expression of endogenous NOTCH1 and caused its accumulation in the endoplasmic reticulum. Also, *in vitro* unfolding assays revealed that an addition of *O*-glucose enhanced stability of a single EGF repeat while an extension

by xylose weakened it. These findings indicate that *O*-glucose glycans are required for proper trafficking of Notch receptors by regulating the stability of EGF repeats. Mammals have two glucoside xylosyltransferases, *GXYLT1* and *GXYLT2*; however, whose biological significance remains unclear, especially whether they are involved in negative regulation of Notch signaling in mammals is not yet determined. To gain the insight into regulation of Notch signaling by xylosyl-extension of *O*-glucose glycans, we generated four HEK293T knockout clones: *GXYLT1* KO, *GXYLT2* KO, *GXYLT1* and *GXYLT2* double KO and *XXYLT1* KO by CRISPR-Cas9 technology. Successful deletion of each gene was confirmed by genomic sequencing of the gRNA-targeted regions. Mass spectrometric analysis of *O*-glucose glycoforms on the mouse Notch1 extracellular domain overexpressed in the knockout clones confirmed complete loss of the xylose residues in *GXYLT1* and *GXYLT2* double KO clone and loss of the second xylose in *XXYLT1* KO clone as expected. Interestingly, our mass spectrometric data showed significant reduction of xylosyl-extension on specific EGF repeats by deleting *GXYLT1*, suggesting distinct roles of two *GXYLTs in vivo* like three mammalian Fringes, known regulators of Notch signaling, that add GlcNAc to *O*-fucose on EGF repeats.Flow cytometric analysis showed that global elimination of xylosyl-extension on *O*-glucose glycans did not change endogenous NOTCH1 expression on the cell surface in contrast to substantial reduction observed in the *POGLUT1* KO clone. Currently we are performing further experiments to grasp the significance of xylosyl-extension on *O*-glucose glycans in Notch signaling. This work was supported by KAKENHI 17H06743 (to H.T.).

(74) Glycoscience mass spectrometry tools funded by the NIH Common Fund for Glycoscience

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The following summarizes recent improvements to our publically available, open-source software tools for interpretation of glycoscience mass spectrometry data.

Our glycoinformatics library (**GlyPy**), has had substantial work done to improve its substructure analysis capabilities to service the needs of multiple groups using the library. It now has the ability to simulate enzymatic synthesis of glycans, allowing it to build collections of glycans de novo in addition to querying external databases or manual construction. It continues to track the development of GlyTouCan to keep up with new features being added to the RDF schema.

Ms_deisotope, our raw mass spectrum processing library, has continued to improve its ability to process complex, highly charged, glycan and glycopeptide LC-MS and LC-MS/MS data. It is capable of competitive deconvolution performance compared to both vendor software and existing academic projects. It can now track precursor purity and average scans to better interpret low-abundance or noisy regions of spectra. GlycReSoft uses ms_deisotope in its deconvoluter pipeline and chromatogram scoring procedure included in the above publication and in the component that we provided to GRITS. This interoperability is possible because of ms_deisotope's ability to write its output in mzML format, a community-driven, open standard maintained by HUPO-PSI for representing raw and processed mass spectrometry data.For **GlycReSoft**, our LC-MS/MS search engine for glycans and glycopeptides, we have improved the ability to identify glycans using the network smoothing. Included in this tool is a renderer for annotated mass spectra, chromatograms, protein glycosite coverage and microheterogeneity, producing summary reports and high-quality vector graphics for presentations. The scoring function and database search mechanism has been updated using recent developments in the field to make it applicable to whole glycoproteome databases. We have also GlycReSoft for application to glycoproteomics of proteoglycans.

We continue to maintain a library of synthetic glycosaminoglycan standards funded by NIH (http://www.bumc.bu.edu/zaia/gag-synthetic-saccharides-available/).

We developed GAGfinder, the first GAG-specific software for automatically detecting and annotating isotopic distributions in GAG tandem mass spectra. Our lab has used GAGfinder for its various GAG projects for spectral analysis (http://www.bumc.bu.edu/msr/software/).

We have converted the PeakInterpreter of our efficient algorithm for accurate de novo glycan topology reconstruction from tandem mass spectra (**GlycoDeNovo**) into Java and have tested it extensively using in-house data. The source codes have been made available to the public (https://github.com/hongpengyu/GlycoDeNovo).

(75) Two key motifs determine topology and polyprenol substrate binding of PglC, a monotopic phosphoglycosyl transferase from Campylobacter jejuni

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Phosphoglycosyl transferases (PGTs) are integral membrane proteins that initiate a wide variety of essential glycoconjugate biosynthesis pathways, including peptidoglycan and N-linked glycan biosynthesis, by catalyzing the transfer of a phosphosugar from a sugar nucleoside diphosphate donor to a membrane-resident polyprenol phosphate. PGTs can be grouped broadly into two superfamilies based on their membrane topology. One superfamily, exemplified by bacterial MraY and WecA, includes polytopic PGTs with 10- and

11- transmembrane helices respectively and active sites crafted from extended cytoplasmic inter-TM loops. The eukaryotic PGT Alg7, which initiates the dolichol pathway for N-linked protein glycosylation, belongs to this superfamily. A second superfamily is exemplified by the monotopic enzyme, PglC, from N-linked protein glycosylation pathway from the Gram-negative pathogen *Campylobacter jejuni*. The PGTs in this superfamily share a common functional core, which is homologous to PglC and comprises a single N-terminal membrane-inserted domain and a small globular domain.

We recently reported the X-ray structure determination of PglC. In the reported structure, the N-terminal domain of PglC forms a reentrant helix-break-helix structure, termed the reentrant membrane helix (RMH), such that both the N-terminus and the globular domain, which includes the active site, are on the cytoplasmic face of the inner membrane. Whereas many membrane-spanning proteins have been structurally characterized and transmembrane topologies can be predicted computationally, relatively little is known about the determinants of reentrant topologies in membrane proteins. The definition of this unique structure has prompted *in vivo*, biochemical, and computational analyses to understand and define two key motifs that contribute to the reentrant membrane topology and to provide insight into the dynamics of the enzyme in a lipid bilayer environment. Using the new information gained from studies on the PGT superfamily we demonstrate that the two motifs exemplify principles of topology determination that can be applied to the identification of reentrant domains among diverse monotopic proteins of interest.

In addition, we explore the structure-function relationship between the RMH domain of PglC and specificity of binding of the membrane-resident polyprenol substrate. We find that correct positioning of the membrane-inserted domain within the PglC structure is necessary for catalytic activity, and identify a conserved proline within the domain that we hypothesize plays a particular role in recognition and possible recruitment of polyprenol substrate in the membrane. The significance of this residue for substrate binding is investigated using a diversity of biochemical methodologies. The work provides a model for how PGTs of this superfamily might identify their polyprenol substrate, which is lowly abundant in the bulk membrane.

(76) Glycan characterization of intact glycoproteins by NMR

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Glycoprotein characterization continues to be a difficult yet ever growing field of biochemistry. Various mass spectrometric methods can be applied to determine the glycosylation sites on proteins and peptides and thus yield the mass of the attached glycan. These methods may not however offer reliable quantitative data related to the absolute amount of glycosylated peptide or non-glycosylated peptide due to the variation in ionization potential of different species. It is also difficult to distinguish between isobaric species in an unknown sample. NMR can offer reliable characterization of isobaric glycan monomers that are isomers at a single stereocenter. A well-known limiting factor in NMR is the large size of proteins which leads to fast relaxation and subsequent loss of signal intensity. While this is true for a globular molecule it is an oversimplification that doesn't take into account the fact that many posttranslational modifications are extensions on the protein molecule and have different NMR relaxation profiles. We present a method to observe glycans on intact glycoproteins at natural abundance. Leveraging the NMR relaxation difference between the glycan and protein residues allows us to clearly see the glycan signals while attenuating those from the protein. This protein signal attenuation is efficiently achieved using a simple HSQC-TOCSY (DIPSI-2) with various mixing times. We have demonstrated the proficiency of this technique using RNase B with varying glycoforms. Commercial RNase B has Man5-Man9 N-glycans and we have also utilized a sample with homogenous Man5 glycans. Reduction of the protein signals greatly simplifies the spectrum making it much easier to locate signals corresponding only to the glycan and not the protein. Not only does the mixing sequence reduce the amount of signal from the protein, but it also yields the coupled spin system from each of the monomer rings in the glycan. While using the HSQC-TOCSY for glycan assignments is well known, it hasn't been explicitly shown to significantly attenuate protein signals or to leverage the relaxation difference to obtain the greatest difference between glycan and protein signal. We hope to apply this method to other glycoproteins with dynamics similar to that of RNase B going forward.

(77) How does glycosylation modulate Skp1 organization?

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Glycosylation diversifies protein structure beyond the scope of the 20 canonical amino acids. The importance of glycosylation is widely acknowledged, but the molecular mechanism by which they modulate protein structure and function remains poorly understood. Here we describe a glycosylation system that regulates protein/protein interactions involving Skp1, which is an essential component of the SCF (Skp1/Cullin-1/F-box) E3 ubiquitin ligase. Skp1 is an adaptor that recruits F-box proteins (FBP) and their target substrates to Cullin-1 for polyubiquitination and subsequent degradation. During complex formation, the F-box binding domain near the C-terminus of Skp1 undergoes a coil-to-helix transition. In protists including the model organism *Dictyostelium* and the agent for human toxoplasmosis

Toxoplasma gondii, a C-terminal hydroxyproline residue of Skp1 can be modified by a highly conserved linear pentasaccharide, resulting in increased formation of the Skp1/FBP subcomplex both in vitro and in vivo. Our NMR studies on Skp1 organization and glycan dynamics, together with all-atom molecular dynamics simulations that satisfy NMR constraints, suggest a cis-acting mechanism by which the glycan encourages local helicity in addition to promoting an open conformation susceptible to interaction with an F-box. However, NMR constraints were collected at a concentration where Skp1 exists as a dimer while MD simulations were conducted on a monomer model. Our recent sedimentation velocity analysis by AUC suggests that Skp1 self-associates into a homodimer with a dissociation constant of 2.5 μM which is near reported physiological concentrations. We will show a new NMR solution structure of Skp1 which reveals how dimer formation competes with F-box binding, and use this as the basis for new MD simulations to evaluate the glycan cis-acting mechanism. These findings are also expected to inform new mutagenesis approaches to examine the significance of Skp1 dimerization with respect to substrate activity for glycosylation, and competition with F-box binding.

(78) Structural Characterization of Glycosylated Proteins by NMR

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Structural characterization of glycosylated proteins by any method is challenging because of the heterogeneity of glycosylation and the extensive conformational sampling by most glycans. For NMR there are extra challenges because expression in mammalian cell cultures, which can preserve near-native glycosylation, requires expensive substrates to achieve uniform isotopic labeling with ^{15}N or ^{13}C, and mammalian cells do not tolerate high levels of deuteration (something required to improve spectral resolution). Here we describe a sparse-labeling approach that preserves high resolution without deuteration, proves to be economical in most applications, and returns sufficient structural data to define geometries of multiple domain proteins and ligand protein complexes. The approach is based on labeling proteins with a single type of amino acid isotopically labeled in methyl groups with ^{13}C (^{13}C-valine or ^{13}C alanine). NMR signals from methyl groups are special in terms of the sensitivity and resolution that they provide. We illustrate that direct detection of ^{13}C as opposed to ^{1}H enhances this inherent advantage. The addition of paramagnetic tags to specific sites in a protein provides long range distance and angular information on the location of labeled methyl groups. In combination with a newly developed approach to assign methyl resonances to specific sites in a protein, this information proves adequate to define relative domain orientations and facilitate the identification of ligand binding sites. Application to terminal domains from cell surface signaling molecules, including ROBO1 and CEACAM1, is being used to illustrate the approach.

(79) Structural basis of glycan counting in glycosyltransferases

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The biosynthesis of the lipid-linked oligosaccharide (LLO) that is used as glycan donor during N-glycosylation, requires the action of a set of membrane-associated glycosyltransferases that catalyze the transfer of glycan units from activated sugars to growing LLO acceptors[1]. Most of them catalyze a single transfer, however, glycosyltransferases catalyzing the addition of multiple glycan units are also found in the pathway. In *Campylobacter jejuni*, the glycosyltransferase PglH is the only enzyme in the LLO biosynthetic pathway that performs multiple glycans addition, it transfers three GalNAc units to the LLO[2]. In eukaryotes, Alg11 perform a similar task, it is responsible for the addition of two Mannose units consecutively during LLO synthesis[3]. We have combined structural studies and molecular dynamics simulations on PglH and found that it contains an amphipathic helix, which has a double function, as a structural element for membrane anchoring and as molecular ruler for glycan counting. This "ruler helix" has a set of positive charge residues that interact with the pyrophosphate group of the LLO during glycan elongation[2]. This mechanism might be shared by other glycosyltransferases from the same family and opens future studies on eukaryotic glycosyltransferases as Alg11.

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(80) N-glycan processing in NMDA ligand binding domain depends on glycan-polypeptide and intradomain interactions

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N-methyl-D-aspartate receptors (NMDAR) are heterotetrameric membrane receptors ubiquitously expressed in the central nervous system that bind neurotransmitters and transport small ions. The extracellular ligand-binding domain (LBD) is heavily glycosylated with highly conserved asparagine(N)-linked glycosylation sites, however the structural and functional roles of these N-glycans are poorly understood due to difficulty in observing these mobile moieties by x-ray crystallography and cryo-electron microscopy. We applied solution- and computation-based methods to identify intradomain and interglycan interactions mediated by N-glycans in the LBD of the GluN1 subunit of an NMDAR that potentially impact NMDAR structure. HEK293 cells expressed [^{13}C-glycan]-LBD that provided clear signals corresponding to each of the three N-glycans (at residues N440, N471 and N771) in 2d NMR experiments. Glycans at N440 and N771 revealed evidence of contact between the reducing end N-glycan residues and nearby amino acids. Mass spectra of the GluN1 LBD N-glycans and N-glycopeptides showed limited core fucosylation of the N471-glycan and reduced branch processing of the N771-glycan compared to the N440 N-glycan. Extensive molecular dynamics simulations identified a manifold of conformations sampled by N-glycan and polypeptide residues. Of note, the distal portions of the N771-glycan contacted the core residues of the N471 glycan, potentially explaining the reduced N471 core fucosylation and N771 branch processing. Furthermore, simulation revealed extensive contacts between nearby amino acids and the N440 and N771-glycans that were consistent with NMR spectra reporting the environment at the reducing ends of these N-glycans. This experimental approach provides evidence that the NMDAR GluN1 LBD N-glycans form specific glycan-polypeptide and glycan-glycan contacts and we discuss a potential role for the GluN1 LBD N-glycans in interdomain contacts formed in NMDA receptors.

(81) Predicting glycan biosynthesis based on enzyme-glycan accessibility

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In this work, computer simulation and crystallographic data are combined to show that glycoprotein glycoform distributions depend on the accessibility of the glycans to the relevant glycosidases in the ER. We illustrate this for three systems: a protein disulphide isomerase named Pdi1, a hemagglutinin (HA) from influenza A, and the HIV envelope protein gp120. In the case of Pdi1p and gp120, glycan-protein interactions modulate glycan processing, whereas in HA, glycan-glycan packing prevents glycan processing.

The presentation will illustrate how site-specific glycan processing can be predicted on the basis of 3D-structure.

(82) Structural and enzymatic studies of Human N-acetylglucosaminyltransferase II (MGAT2)

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Asn-linked oligosaccharides are extensively modified during transit through the secretory pathway, first by trimming of the nascent glycan chains to remove Glc and Man residues and subsequently by initiating glycan branching from the tri-mannosyl glycan core through the action of GlcNAc tranferases. The glycans are subsequently extended to form complex antennae by the action of Golgi glycosyltransferases. The trimming and branching pathway steps are highly ordered and hierarchal based on the precise substrate specificities of the individual biosynthetic enzymes. Mannose trimming to a $Man_5GlcNAc_2$-Asn intermediate structure is followed by β1,2GlcNAc addition to the Man α1,3Man-arm by MGAT1. This reaction forms a GlcNAc β1,2Man α1,3Man- substructure as a "recognition arm" that is essential for recognition by several subsequent processing enzymes. Further mannose trimming by Golgi α-mannosidase II and subsequent β1,2GlcNAc branching by MGAT2 both require prior MGAT1 action for substrate recognition. In an effort to understand the structural basis of complex type glycan biosynthesis, we determined the structure of human MGAT2 as a Mn^{2+}-UDP donor analog complex and as a $GlcNAcMan_3GlcNAc_2$-Asn acceptor complex to reveal the structural basis for substrate recognition and catalysis. The enzyme exhibits a GT-A Rossmann-like fold that employs conserved divalent cation-dependent substrate interactions with the UDP-GlcNAc donor. MGAT2 interactions with the extended glycan acceptor are distinct from other related glycosyltransferases. These interactions are comprised of a catalytic subsite that binds the Man-α1,6-monosaccharide acceptor and a distal exosite pocket that binds the GlcNAc-β1,2Man-α1,3Manβ- substrate "recognition arm". Recognition arm interactions are surprisingly similar to the enzyme-substrate interactions for Golgi α-mannosidase II, despite the fact that the enzymes have completely different structural folds and different mechanisms of action. These data suggest that substrate binding by MGAT2 employs both conserved and convergent catalytic subsite modules to provide substrate selectivity and catalysis. More broadly, the MGAT2 active site architecture demonstrates how glycosyltransferases

create complementary modular templates for regiospecific extension of glycan structures in mammalian cells.

(83) Structure and substrate specificity of human β1,3-N-acetylglucosaminyltransferase 2 (B3GNT2)

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Poly-N-acetyl-lactosamine (poly-LacNAc) structures are comprised of terminal glycan repeat extensions (R-[Galβ (1,4)-GlcNAcβ (1,3)]$_n$-R) on cell surface and secreted *N*- and *O*glycoproteinsand glycolipids. The linear poly-LacNAc repeats are ligands for endogenous lectins and act as scaffolds for additional glycan modifications with roles in development, immune function and human disease. Poly-LacNAc structures are extended by the alternating action of β1,4-galactosyltransferase (B4GALT) and β1,3-*N*-acetylglucosaminyltransferase (B3GNT) enzyme activities. Multiple B4GALT and B3GNT isoforms contribute to poly-LacNAc extension in different tissues and on different glycan classes, but the major *N*- and *O*-glycan poly-LacNAc synthase in most tissues is β1,3-N-acetylglucosaminyltransferase 2 (B3GNT2). To identify the molecular basis for B3GNT2 action, the catalytic domain was expressed in HEK293s (GnTI-) cells as a GFP-fusion protein in the presence or absence of metabolic incorporation of selenomethionine (SeMet). Fusion tags and glycans were cleaved by concurrent digestion with TEV protease and endoglycosidase F1 and the resulting protein was crystallized in the presence of the donor analogue UDP and Mg^{2+}. The structure was solved at 1.55 Å resolution using single-wavelength anomalous diffraction and a subsequent structure was solved as a UDP-Mg^{2+}:lacto-*N*-neotetraose donor and acceptor analogue complex. The structure of B3GNT2 displays a GTA fold containing a *DxD* motif employed for divalent cation-dependent interactions with the UDP-GlcNAc sugar donor. A distal loop region is employed for binding the terminal Galβ (1,4)-GlcNAc- (LacNAc) unit of the glycan acceptor to position the galactose C3-OH as the catalytic nucleophile in the GlcNAc transfer reaction. Modeling of the GlcNAc donor sugar along with kinetic analysis of active site mutants provide evidence for the inverting catalytic mechanism. Comparisons with other related inverting GT-A fold glycosyltransferases demonstrate conserved interactions with the sugar-nucleotide donor, but divergent mechanisms for positioning acceptor structures that facilitate synthesis of diverse monosaccharide linkages by the respective enzymes. The resulting B3GNT2 structural model provides insights into the enzymatic template for elongation of poly-LacNAc chains in animal tissues.

(84) Human Core 3 beta3 N-Acetylglucosaminyltransferase Possesses Novel beta3 N-acetylgalactosaminyltransferase Activity

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Mucin-type O-glycosylation (O-glycosylation) is one type of major protein glycosylation. So far, eight O-glycan core structures, Core 1-8, are known to exist in animal cells. O-glycans based on the Core-1 structure, Galß1,3GalNAc-a-R are the predominant ones found in all animal cells, while O-glycans derived from Core-3 structure, GlcNAcß1,3GalNAc-a-R are strictly synthesized in intestinal epithelial cells. Notably, Core-1 O-glycans on glycoproteins engage in many biological processes, while the Core-3 O-glycans suppress colorectal tumorigenesis and tumor progression. Among the known glycosyltransferases for O-glycan biosynthesis, Core 3 N-acetylglucosaminyltransferase (C3GnT, ß3GnT6) responsible for synthesizing the Core-3 structure is a unique enzyme, but is not well-characterized biochemically. While studying the recombinant human C3GnT, we unexpectedly identified its novel ß3 N-acetylgalactosaminyltransferase (ß3GalNAcT) activity. Evidenced by the data from Mass Spectrometry(MS), UPLC and NMR, while the product synthesized by recombinant C3GnT using UDP-GlcNAc was confirmed to be the Core 3 structure, the product of C3GnT using UDP-GalNAc as its donor was identified as GalNAc**ß1,3**GalNAc-a1-R which is a novel structure, termed Core-9. Interestingly, glycopeptide MUC2-$Tn_{6\sim7}$, but not MUC1-Tn_3, served as an acceptor for the C3GnT in the presence of UDP-GalNAc to synthesize Core-9, although both glycopeptides were utilized as acceptors by the enzyme using UDP-GlcNAc to make Core-3 products, suggesting that the Core-9 O-glycans may only exist on certain mucins. Taken together, these results demonstrate that the C3GnT has a novel ß3GalNAc-transferase activity, in addition to its ß3GlcNAc-transferase activity. This is the first example for a single-functional glycosyltransferase to possess a capability of transferring two different donor substrates to form two different structures.

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

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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 that while all had a strong binding preference for terminal and internal Galβ1-4GlcNAc (LacNAc), there were differences in preference between the C-terminal CRD and N-terminal CRD. Binding assays with the C-terminal mutant and isolated N-terminal and C-terminal CRDs indicate differences in binding preferences as well for epithelial cells and 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. Additional studies are ongoing to examine the role of mucus in IHNV attachment and entry. Secreted by epidermal goblet cells, the mucus layer is continuously shed and replaced, often acting as the first line of defense for fish against pathogens. Mucus is composed of a mixture of glycoproteins, polysaccharides, protein, lipids, salts, and water. Galectins present in the mucus may cross-link IHNV to the mucus glycans, either preventing or facilitating viral entry. 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].

(86) Blocked O-GlcNAc cycling disrupts hematopoietic stem cell homeostasis

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Glutamine, glucose, and other metabolites drive the hexosamine biosynthetic pathway (HBP) ultimately leading to the *O*-GlcNAc modification of critical intracellular targets. Maintanence of *O*-GlcNAc cycling is essential for cellular metabolic homeostasis and stem cell viability. The multipotent hematopoetic stem cell (HSC) is an adult stem cell that relies on glycolysis and glutaminolysis to produce multiple blood lineages. While the role of *O*-GlcNAc cycling has been investigated in embryonic stem cells (ESC), its impact on adult stem cell biology has been largely unexplored. Here, we used genetic deletion of the enzyme that removes *O*-GlcNAc, *O*-GlcNAcase (OGA), to determine the consequences of blocked O-GlcNAc cycling on HSCs. We demonstrated inefficient differentiation of *Oga* deletion ESCs toward the hematopoietic lineage. Further, *Oga* deletion in mouse HSCs resulted in greatly diminished progenitor pools and nearly complete loss of competitive repopulation capacity. Transcriptional analysis of mutant hematopoietic progenitor cells indicated deregulation of key genes involved in adult stem cell maintenance and lineage specification. These findings suggest that *O*-GlcNAc cycling plays a critical role in supporting HSC homeostasis.

(87) CarbArrayART: Carbohydrate Array Analysis and Reporting Tool New software for glycan array for data processing, storage and presentation

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In 2009, the Glycosciences laboratory at Imperial College developed an in-house software suite for storage, processing and presentation of carbohydrate microarray data[1].This has been the mainstay of data management presentation and reporting.

Starting in 2016 we re-implemented the software into a new software suite, CarbArrayART (Carbohydrate Array Analysis and Reporting Tool), to make available to the scientific community. The new software is based on GRITS Toolbox[2] which was originally developed for processing,

interpretation and archiving of glycomic mass spectrometry data. GRITS contains functions for storing glycan structures and also metadata, such as project information, sample description and experiment details. We are capitalizing on these functions by developing and implementing CarbArrayART as an extension (plugin) of GRITS Toolbox.

The main features of CarbArrayART are (1) storing carbohydrate microarray data from different array formats. Users are able to store the results (scan data) of the binding assay results, e.g. Proscan or GenePix files, and related array-specific metadata, such as glyco-probe lists, array geometry, sample (glycan binding systems) information and experiment protocol information. (2) Presentation of data with filtering and sorting functions. Tables and charts are generated automatically based on the stored scan data, the corresponding array geometry, and the filter and sorter applied. (3) Reporting of data. In addition to the original function of GRITS Toolbox in generating reports automatically with sample information and experimental protocol metadata, we implement a function to generate reports conforming to MIRAGE guidelines for glycan microarray-based data[3].

A prototype of CarbArrayART was presented at the Bioinformatics satellite of the SFG meeting 2017. Since then we rendered it more flexible by developing several new data input systems for carbohydrate microarray associated data and metadata. These include metadata for carbohydrate-binding samples, microarray experiments and array geometry for multiple glyco-probe layouts. These are all MIRAGE compliant. We are updating data presentation systems to include customizable array geometry and reporting systems in Word and PDF format to display glyco-probe lists.

Supported by Wellcome Trust

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(88) Microarray approach to detect novel biomarkers that stratify the pathogenesis of sepsis

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Recent findings by this laboratory both in press and published have identified glycosidase–mediated N-glycan remodeling of relevant blood glycoproteins in modulating protein half-lives and abundance in the pathogenesis of sepsis involving mechanisms that appear remarkably distinct in the onset of sepsis caused by different pathogens. Our findings are the first to implicate and identify multiple pathogenic pathways in sepsis with the potential to stratify and diagnose the origins and progression of sepsis beyond that of the generally accepted view that sepsis reflects a singular disease mechanism. To investigate the potential to identify unique protein and glycan biomarkers specific to sepsis caused by different bacterial pathogens, we have first designed and implemented an antibody microarray that includes over 60 target proteins comprising multiple cytokines, various inflammatory mediators, and other physiologically relevant blood glycoproteins to determine whether these circulating targets are differentially modulated by the host in sepsis caused by different pathogens. Microarray chip-based assays allow for a more rapid and comprehensive analysis of the disease process by interrogating dozens of targets simultaneously. Our strategy further includes a comparative analysis of both Gram-positive and Gram-negative pathogens isolated from human sepsis patients with incorporation of mouse models of sepsis wherein genomic responses have been shown to closely correlate with those in human sepsis. Our ongoing studies interrogate blood plasma and sera isolated from septic mice and human patients diagnosed with severe sepsis or septic shock. The results of antibody microarray studies currently ongoing will be presented, with data that identifies both common and unique changes to the circulating blood proteome that indicate the presence of unique pathogenic processes. These differences may provide the leverage needed to identify the origin and onset of sepsis at earlier time points than can be assessed by current clinical diagnostics.

(90) N-glycosylation is involved in the maturation of brain-derived neurotrophic factor

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Brain-derived neurotrophic factor (BDNF) is the most abundant brain neurotrophin that plays essential role in the development and maintenance of the central nervous system while its dysregulation accompanies number of neuropsychiatric disorders. In line with its essential function, the production of BDNF is highly regulated. BDNF is synthesized as a pro-protein (proBDNF) activated to the mature form by proteolytic cleavage by furin-type convertases and sorted into secretory or endolysosomal pathways through interactions with sortilin. ProBDNF carries a single conserved N-glycosylation sequon located 8 aa upstream of its convertase cleavage site. Our experiments show that the N127 is glycosylated and the LC-MS/MS analysis of tryptic glycopeptide 121NYLDAANMSMR revealed, for the first time, that proBDNF carries N-glycans terminated by GalNAcβ1-4GlcNAcβ1-R (LacdiNAc) and modified to a large degree by fucosylation at various positions and 4-O-sulfation at the terminal GalNAc. Wild-type proBDNF and non-glycosylated N127Q mutant were expressed in human 293 F cells. Mutation of the sequon prevented secretion of (pro)BDNF and proBDNF

was retained in cells in full length form indicating a function of the N-glycosylation in proteolytic cleavage or intracellular sorting. Deglycosylation of purified proBDNF with PNGase F increased its binding to immobilized sortilin approximately 4 times in ELISA based assay. Moreover, deglycosylation of sortilin further enhanced this interaction strongly suggesting regulatory role of N-glycans. At present, we study proteolytic cleavage of proBDNF by furin using a competition assay with glycosylated and deglycosylated proBDNF forms differentially labeled with Cy3 and Cy5 dyes. It is expected that this overlooked glycosylation of the prodomain of BDNF represents a novel regulatory element in maturation and intracellular targeting of (pro) BDNF. Further studies are needed to explore this regulatory pathway and its potential targeting for therapeutic interventions in disorders associated with the dysregulation of BDNF.

(91) Metabolic regulation of KLHL proteins through O-glycosylation

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The Kelch-like (KLHL) family of proteins are critical substrate adaptors for E3 ubiquitin ligases and mutations in KLHL genes underlie a range of human diseases, including cancer and neurodegeneration. However, the upstream regulation and downstream effects of most KLHL proteins remain poorly understood. The best-characterized KLHL protein is KEAP1, whichbinds to the CUL3/RBX1 E3 ligase components to mediate the ubiquitination and destruction of NRF2, a master transcriptional regulator of redox stress signaling.Redox-active compounds disable KEAP1, preventing the ubiquitination of NRF2 by KEAP1/CUL3/RBX1 and allowing NRF2 accumulation and activation.We have demonstrated that KEAP1 is modified by O-GlcNAc, a nutrient-sensitive, reversible form of glycosylation, and this modification is required for efficient NRF2 ubiquitination and degradation. Blockade of KEAP1 O-GlcNAcylation by chemical inhibition or nutrient deprivation stabilizes and activates NRF2, revealing a new regulatory link between nutrient sensing and redox stress signaling. Interestingly, we recently discovered that the KLHL protein gigaxonin is also O-GlcNAcylated in a nutrient-dependent manner.Gigaxonin is a component of E3 ubiquitin ligase complexes that promote the normal proteolytic degradation of intermediate filament proteins in neurons and other tissues.Mutations in gigaxonin cause intermediate filament aggregation and neuronal dysfunction, resulting ingiant axon neuropathy, a fatal neurodegenerative disease.Our preliminary data indicate that site-specific, nutrient-responsive O-GlcNAcylation of gigaxonin may be required for its function in promoting intermediate filament protein turnover. Overall, these studiesindicate that glycosylation of KLHL proteins may be a general regulatory link between metabolic status and proteostasis in a wide variety of pathopthysiological contexts.In the long term, our results may point to new therapeutic opportunities inneurodegeneration, cancer and other diseasesby manipulating KLHL proteins through O-GlcNAc modulation.

(92) Evolution of Glycan Mediated Cross-linking Interactions

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We recently provided evidence for a family of glycan tumor antigens that possess carbohydrate-carbohydrate interactions (1). These include Tn (GalNAc1-Ser/Thr), STn (Neu5Ac2,6GalNAc1Ser/Thr), LewisX and LewisY. Interestingly, all of these carbohydrates are onco-fetal antigens. Lectin-carbohydrate cross-linking interactions are also known to be involved in embryogenesis and oncogenesis. Furthermore, the ability of lectins to form homogeneous and heterogeneous cross-linked complexes with multivalent carbohydrates (2-4) appears to be mirrored by carbohydrate-carbohydrate interactions. We suggest that carbohydrate-carbohydrate interactions are a primitive form of glycan mediated cross-linking interactions that may have preceded lectin-carbohydrate cross-linking interactions.

(93) Mucin glycosylation pathways in human eye cells

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Highly O-glycosylated mucins in the epithelial glycocalyx and tear fluid have an important barrier function protecting the eye from adverse environmental conditions such as dehydration, injury and infection. N-glycans are also found on glycoproteins of the eye surface but their functions are poorly understood. To understand the biochemical basis of glycans of the human ocular surface, several biochemical and molecular biology approaches have been taken. Despite these efforts, however, the glycosyltransferase (GT) activities responsible for the synthesis of O- and N-glycans have not yet been determined in ocular cells.

We have cultured human corneal and conjunctival epithelial cells with the goal to establish GT activities involved in O- and N-glycosylation. The results indicate that corneal epithelial cells have active enzymes that synthesize O-glycans with sialylated core 1, Galβ1-3GalNAcα, and core 2, GlcNAcβ1-6 (Galβ1-3)GalNAcα structures, but not core 3 and 4 structures. The activities of GTs help to understand the prevalence of specific glycan structures found on the surface of the eye. The enzymes were similar in conjunctival epithelial cells.

High levels of proinflammatory cytokines have been associated with many ocular surface disorders, such as dry eye, a disease affecting millions worldwide. Treatment of corneal cells with TNFα resulted in changes of GTs activities involved in O-glycosylation and in the extension of both N- and O-glycans. Taken together, these data indicate that theproinflammatory cytokine TNFα influences the expression

of GTs and change glycosylation in a dynamic fashion during inflammatory stimuli of the ocular surface.

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(94) Function of Protein O-mannosyltransferases 1/2 in connectivity of sensory neurons and control of muscle contractions in Drosophila

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Protein O-mannosyltransferases 1 and 2 (POMT1/2) are best-studied enzymes that modify Ser/Thr residues of proteins with O-mannose. They modify Dystroglycan and play particularly important role in muscle and neuronal physiology and several congenital muscular dystrophies are caused by defects POMTs and other enzymes involved in protein O-mannosylation (POM). POMT1/2 are well conserved among metazoan organisms, from fruit flies to humans. Despite several studies on POMT1/2, mechanisms of their normal functions and pathological mechanisms associated with their mutations are not fully understood. Therefore, we use Drosophila as a model system to shed more light on their developmental functions and cellular pathomechanisms that cause the disease. *POMT1* and *POMT2* genes are known in Drosophila as *rotated abdomen* and *twisted*, respectively, because their defects result in the conspicuous developmental phenotype of 'rotation' of abdominal segments in adult flies. We found that *POMT* mutants also develop an analogous body torsion defect at embryonic stages. The mutants show a gross morphological abnormality in alignment of body segments that becomes prominent during peristaltic waves of muscle contractions. We found that the pattern of muscle contractions is significantly affected in POMT mutants. We theorized that the abnormal contractions are caused by defects in communication between sensory neurons and the central nervous system, thus resulting in a failure to maintain normal body posture and leading to the body torsion phenotype. We tested this hypothesis by detailed in vivo analyses of muscle contractions and by characterization of the patterning of sensory axon terminals in the ventral ganglion. We applied MARCM and FLP-out strategies to examine cell autonomous requirement of POMTs for sensory axon wiring. Using genetic and cell biological approaches, we revealed a novel function of POMTs to maintain communication between PNS and CNS. Therefore, we posited that this function is conserved in mammals and might contribute to pathological mechanisms of muscular dystrophies in humans, which potentially can open up new avenues for developing therapies. Our current studies are aimed at uncovering genetic and molecular pathways that underlie POMT function in controlling neural communications and muscle contractions. We will present new data that shed light on functional targets of POMTs in these processes. This project was supported in part by NIH/NS075534, NS099409 and CONACYT 2012-037(S) grants to VP.

(95) Glycomic Analysis Of Host-Response To Influenza In A Model Of Young Children And Adults

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Influenza viruses cause severe respiratory illness and worldwide human deaths. Glycosylation plays a critical role in influenza severity and virus pathogenesis. Both viral strain and host factors can impact the severity of influenza, however, the major molecular drivers to predict the outcomes of influenza infection remain unknown.

Our study is focused on understanding the role of host glycosylation in influenza response and the role age might play in severity. Using a ferret model, we compare responses in adult and newly weaned animals, a model for adult humans and young children, respectively. We are studying response to the novel H1N1 2009 pandemic virus, in which young adults were at higher risk than either the elderly or young children for a severe response. Glycomic analysis using our lectin microarrays identified changes in glycosylation in the adult host lung that stratify by severity. Based on the observe glycomic response and other data, we propose a mechanism in which presentation of key glycan epitopes over-activates the immune system, causing the differential severity in adults. We are currently testing whether glycomic differences in response between adults and young children to the H1N1 2009 pandemic virus might explain the differential susceptibility to pandemic influenza infection of adults.

(96) Streptococcus pyogenes evades adaptive immunity through specific IgG glycan hydrolysis

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The human specific pathogen *Streptococcus pyogenes* secretes EndoS, an endoglycosidase with activity on the conserved N-glycan on human IgG. This glycan hydrolysis impairs IgG effector functions *in vitro*, but it is still unclear how it contributes to pathogenicity during infections.

In a cohort of patients with *S. pyogenes*infections (mild to severe) we analyzed IgG glycan hydrolysis using selected reaction monitoring (SRM) targeted mass spetrometry. This revealed substantial IgG glycan hydrolysis locally at infection sites and systemically in the patients with septic shock. Level of glycan hydrolysis clearly correlated with disease severity. To validate these findings, we set up a mouse infection model with naive and immunized mice that were infected with wild type and *ndoS* knockout bacteria. This revealed that *ndos* knockout bacteria were attenuated

compared to wild type in immunized, but not naive mice, and that the level of IgG glycan hydrolysis correlates with bacterial burden. Furthermore, *ndoS* knockout bacteria were attenuated in IgG dependent *in vitro* assays of phagocytic killing.

This study for the first time shows bacterial specific hydrolysis of IgG glycans during infections in humans and links this to virulence in an animal infection model. This has clear implications for treatment of *S. pyogenes* infections, and especially for future efforts at vaccine development. Since other pathogenic bacteria have enzymes related to EndoS, it is also plausible that this mechanism of immune evasion is not restricted to *S. pyogenes*.

(97) Lack of Glucosidase I Results in Cell Toxicity That Is Relieved By Avoiding the Accumulation of Triglucosylated Glycoproteins

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Glucosidase I (GI) is an endoplasmic reticulum (ER) membrane protein that removes the outermost glucose from the *N*-glycan $Glc_3Man_9GlcNAc_2$ (G3M9) transferred to proteins. Mutations in the GI-encoding gene (*gls1*) result in congenital disorders of glycosylation (CDG) IIb. Even though it has been previously reported that GI absence is lethal in the fission yeast *Schizosaccharomyces pombe*, we were able to obtain two viable *Δgls1* mutants: one with a very sick but non-lethal phenotype (*Δgls1*-S) and the other with a much healthier one (*Δgls1-H*). While the sick strain only displayed G3M9 as ER protein-linked glycan, the healthier strain had not only G3M9 but also $Man_9GlcNAc_2$. The most abundant lipid-linked glycans formed were G3M9 and $Glc_2Man_9GlcNAc_2$ in the sick and healthy mutants, respectively. This result suggested a reduced *alg10+* gene product activity in the *Δgls1-H* strain (Alg10p is responsible for the addition of the last Glc during the step-wise synthesis of lipid-linked G3M9). A mutation in the *alg10+* gene was indeed observed upon sequencing the *alg10* gene in the *Δgls1-H* strain. We constructed a *Δgls1/Δalg10* double knock out mutant and, as expected, this strain showed an almost normal phenotype. Our findings indicate that abrogating G3M9 deglucosylation was responsible for the severe defects observed in *Δgls1-S* cells. Further results demonstrated that such defects could not be ascribed to hindrance of glycoprotein entrance into calnexin quality control of glycoprotein folding cycles, to inhibition of the oligosaccharyltransferase by the transfer reaction products, or to a reduced degradation of misfolded glycoproteins. Moreover, lack of triglucosylated glycoprotein deglucosylation did not significantly prevent Golgi glycan elongation or modify the overall cell wall monosaccharide composition. Nonetheless, GI absence produced a distorted cell wall and absence of the ER underlying structures that occur in *S. pombe* WT cells. We propose that accumulation of G3M9-bearing glycoproteins is at least partially responsible for the defects observed in the CDG IIb disorder.

(98) Towards a novel cancer vaccine: Characterisation of the glycome of canine melanoma cells

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Aberrant glycosylation is a classical hallmark of human cancer. It is well established that the structures and expression levels of polylactosamine-containing glycans are greatly altered during malignant transformation and tumour progression. Such glycans have been implicated in the trafficking of tumour cells and the suppression of both the innate and adaptive immune response. We have recently shown that the transformation of human melanocytes to malignant melanoma cells is associated with the conversion of branched polylactosamines (the I-antigen) to linear polylactosamines (the i-antigen)[1]. The latter are established ligands for galectins.

Cancers in dogs are considered to be excellent models for analogous cancers in humans. However virtually nothing was known about glycosylation in canine melanoma cells. In the current study, we have employed MALDI-TOF and GC-MS glycomic methodologies to analyse the N-, O- and glycolipid glycomes of melanoma cells from a stage III canine melanoma patient. We have found that canine melanoma cells express a repertoire of glycans that are similar to those associated with human melanoma cells, except that, as expected, some of the glycans are capped with N-glycolylneuraminic acid and Gala1-3Gal sequences. Both human and canine melanoma cells express gangliosides GD2 and GD3 that are known ligands for Siglec-7, an immune signal-transducing receptor that inhibits natural killer (NK) cells. They both also express elevated levels of linear polylactosamine sequences. Thus canine melanoma cells express glycoconjugates that have previously been implicated in the suppression of both the adaptive and innate immune response[2].

We hypothesised that the global inactivation of immunosuppressive carbohydrate sequences from tumour cells would result in a greatly enhanced anti-tumour immune response. A pilot clinical trial, following up on this hypothesis, is in progress at the University of Missouri in collaboration with veterinary oncologist Jeffrey Bryan. Preliminary results suggest that deglycosylated autologous vaccines induce a tumour-specific cytotoxic response in dogs with oral melanoma. The progress of this trial will be reported.

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(99) Sox2 drives ST6Gal-I expression and activity to promote a CSC phenotype in ovarian cancer

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This study elucidates ST6Gal-I, a sialyltransferase, as a functional driver of a cancer stem cell (CSC) phenotype regulated by the stem cell transcription factor Sox2. ST6Gal-I is upregulated in 98% of ovarian cancers (OC) and its high expression correlates with reduced overall and progression free patient survival in high-grade serous ovarian carcinoma. ST6Gal-I adds an a2-6 sialic acid to *N*-glycosylated proteins bound for the cell surface, and through this activity, ST6Gal-I modulates the function of key receptors that control cell phenotype. Normal differentiated epithelia have very low expression of ST6Gal-I, however, expression is turned on in stem cell compartments and transformed tissues. Furthermore, studies from our group have shown that ST6Gal-I plays a causal role in conferring hallmark CSC properties including tumor-initiating potential, promotion of tumor recurrence, and resistance to tumor-associated stressors like chemotherapies, serum deprivation, and hypoxia. However, despite compelling evidence for ST6Gal-I's involvement in carcinogenesis, very little work has been done to identify the transcriptional drivers of ST6Gal-I expression. Intriguingly, Sox2 and ST6Gal-I are both located on one of the most commonly enriched amplicons in human cancer, amplicon 3q26. We examined TCGA databases and found that these two genes have coordinate increases in gene copy number in 48/73 cancer cohorts, including ovarian cancer. Furthermore, in those 48 cohorts with co-amplification, there is significant co-occurrence per individual patient samples. Similar to the TCGA cohorts, we evaluated the NCI-60 panel of human cancer cell lines, and found a highly significant positive correlation between Sox2 and ST6Gal-I copy number amplification. In addition to being genetically co-amplified, our work suggests that Sox2 binds directly to the ST6Gal-I promoter to upregulate ST6Gal-I expression. We identified multiple Sox2 response elements in the ST6Gal-I promoter and performed a chromatin immunoprecipitation (ChIP) assay to confirm Sox2 binding. We then overexpressed or knocked down Sox2 in ovarian cancer cells, and found that high expression of Sox2 directly induces ST6Gal-I mRNA and protein expression. Moreover, Sox2-mediated ST6Gal-I upregulation leads to increased surface α2-6 sialylation. Finally, given the known roles of Sox2 and ST6Gal-I in promoting a CSC phenotype, we profiled the expression of stemness markers Oct4, Nanog, and c-myc. Sox2 overexpression or knockdown resulted in up- or down- regulation of Oct4, Nanog, and c-myc, respectively. Importantly, ST6Gal-I knockdown in cells with high Sox2 expression inhibited the Sox2-induced expression of stemness markers, suggesting that ST6Gal-I upregulation is required for Sox2-dependent CSC re-programming. Taken together, these results suggest that ST6Gal-I acts as a critical downstream effector of Sox2 in imparting CSC characteristics that drive tumor recurrence and metastasis.

(100) Creation of activity-verified arrays of mammalian glycan-binding receptors for screening of microbial ligands

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A novel lectin array format is being developed using carbohydrate-recognition domains (CRDs) from mammalian C-type lectins modified with single-site biotin tags, thus avoiding potential confounding effects of appended Fc domains or chemical immobilization. Biotin-tagged CRDs are created by encoding a biotinylation sequence, based on the natural target for biotin ligase in pyruvate oxidase, in bacterial expression systems for the CRDs. The tag is biotinylated during expression in the bacterial by co-expression of biotin ligase. Structural analysis shows that the C-terminal biotin tag projects from the opposite site of the CRD compared to the sugar-binding site. The tag is accessible to streptavidin binding and the CRDs have been extensively characterized in binding assays with glycans and glycoproteins. The tag allows immobilization on streptavidin in a 96-well format or on glass slides in a microarray format. Key advantages of this approach are that the CRDs can be abundantly expressed in bacterial systems, they are stable, and purification is by affinity purification on sugar ligands, which ensures that the proteins are fully active. This feature gives confidence in negative as well as positive results. The focus for development of the array has been on receptors likely to bind pathogens, particularly transmembrane receptors and the soluble collectin group. In some cases, extended portions of the extracellular domains of receptors have also been created, with biotin tags appended at the N-terminus, to preserve the natural oligomeric structures of receptors such as DC-SIGN tetramers and langerin trimers. Model arrays have been generated for both human and bovine receptors. Initial experiments indicate robust binding of yeast zymosan as a model particulate ligand.

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

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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. Lately, we identified a second galectin (CvGal2) with four tandemly-arrayed carbohydrate recognition domains (CRDs) close related to CvGal1. The glycan array analysis revealed that CvGal2 has broader binding specificity for ABH blood group oligosaccharides, 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. Nevertheless, 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 β-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 s*pecies by the oyster and clam lectins 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. We successfully isolated a novel galactosyl-binding lectin 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 lectins. Glycan array analysis, however, revealed that MaGal1 preferentially binds to Gal(α1-3/4) Gal(β1-3/4) GlcNAc, unlike both CvGal1 and CvGal2 that prefer ABH blood group oligosaccharides. Consistently, it preferentially binds to asiofetuin over PSM, the preferred ligand for CvGal1 and CvGal2. Microbial array analysis revealed that it binds strongly to *Shigella dysenteriae* type 1 and two types of *Streptococcus pneumoniae* (type 6B and 15B). Except type 6B and 15B, MaGal1 binds weakly to several other *S. pneumoniae*-types, which is consistent with its ligand preference. Current studies are aimed at the structural basis for the CvGal1, CvGal2, and MaGal1, differential recognition *Perkinsus* species. [Supported by grants IOS-0822257, IOS-1063729, and IOS-1656720 from NSF, and grant 5R01GM070589-06 from 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].

(102) Thermodynamic Analysis of Galectin-1, -3 binding to β-galactosides and MUC1-TF bearing Glycopeptides

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Recognition of cell surface glycans by endogenous glycan-binding proteins, known as lectins, mediates many important aspects of cell-cell recognition, and as such they are integral to many disease processes, including inflammation and tumor cell metastasis. The Thomsen-Friedenreich (TF) tumor-associated antigen, a disaccharide (Gal-GalNAc), has been found in a vast majority of tumor cell types, often occupying the extracellular surface of the transmembrane protein MUC1. Given the evidence that human galectins can interact with mucins and also weakly with the free tumor-associated TF antigen, we have studied the interaction between the galectin-1, -3 and TF antigen presented by MUC1, as a step to characterize the molecular origin of the high selectivity of endogenous lectins to their natural counter-receptors. We have used isothermal titration calorimetry (ITC) to determine the thermodynamic parameters of the binding interaction: binding affinity (K_a), enthalpy change (ΔH), entropy change (ΔS), Gibbs free energy change (ΔG), and the stoichiometry (n). In addition, the thermodynamic binding profile was examined to gain better insight into the driving force involved in the binding interaction between galectin-1, -3 and TF-bearing ligands. We have found that through an enthalpy-driven binding interaction, galectin-3 exhibits higher affinity for the unbound carbohydrate ligands than does galectin-1. Conversely, a thermodynamic switch to an entropy-driven binding interaction was observed for galectin-3 binding to TF-bearing MUC1 analogues. Significant differences were observed in the affinity of galectin-3 versus galectin-1 for the TF antigen, wherein galectin-1 failed to recognize the TF glycan, as well as the TF glycan bound to MUC1 glycopeptides. Thus, the observed selectivity amongst galectin-1 and galectin-3 highlights the importance of studying their binding interactions at the molecular level. Using synthetic glycopeptide models, an in-depth analysis of the binding interactions will assist in identifying inhibitors that can be used as probes for dissecting the mechanisms implicated in galectins' enormously complex and pattern-dependent biological functions. Our long-term goal is to potentially use galectin inhibition to develop therapeutic agents to modulate tumor metastasis and inflammatory diseases.

(103) Elucidation of Mannose Binding Specificities of C-type Lectins Using a Sequence-defined OligoMannose array

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C-type lectins (CTLs) are characterized by the presence of a highly conserved CTL domain that can mediate binding to a variety of glycoconjugates via their carbohydrate moiety of the molecule. However, they differ significantly in the types of glycans that they recognize with high affinity. Among them, a large group of CTLs have been reported to bind to mannose-containing glycans, including DC-SIGN, mannose receptors, langerin and CLEC2. They play vital roles as adhesion and signalling receptors that are involved in homeostasis, pathogen recognition and clearance, and immune cell trafficking and interactions. However, the fine binding specificities of many of those CTLs are elusive due to lack of a collection of sequence-defined mannose-containing glycan standards. Here we report the generation of an oligomannose array using 85 sequence-defined glycan standards. These include 20 linear and 53 branched oligomannose structures, as well as 12 high mannose type *N*-glycan fragments. Six of the branched oligomannose contain phosphate groups on either 6- or 4-position of the terminal mannose. There are, in addition, more than 30 asparagine-linked complex-type *N*-glycans as controls. Using this array, we have analysed the binding specificities of recombinant CTLs that are known to bind to mannose epitope, including human DC-SIGN, L-SIGN, mannose receptors, Dectin-2, langerin, mannose binding lectin, and mouse SIGN-Rs. We anticipate that this data will provide valuable information to the binding preference of the CTLs and lead to new hypotheses about their naturally occurring ligands. In addition, using those glycans as standards, we established mass spectrometry methods to distinguish the isomers of oligomannose using fragmentation information generated by negative-ion mode electrospray coupled with high collision dissociation mass spectrometry. Unique fragmentation patterns at MS^2 were identified attributing to various types of mannose linkages. Our methods enables the quick identification and linkage analysis of naturally occurring oligo- and high-mannose glycans. [This work was supported by NIH Grant P41GM103694 to RDC. We thank Omicron Biochemicals, Inc. for providing many of the *N*-glycans.]

(104) New therapeutic avenues for NGLY1 deficiency

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N-Glycanase 1 (NGLY1) deficiency is a rare inherited congenital disorder caused by heterozygous inactivating mutations in the *ngly1* gene. 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.

Recently, we have linked the absence of NGLY1 to the inadequate activation of the N-glycosylated transcription factor Nuclear Factor Erythroid-2 Related Factor 1 (NFE2L1, also called Nrf1), a member of the "cap'n'collar" (CNC) bZIP family. Nrf1 is involved in many vital metabolic pathways, including the transactivation of antioxidant enzymes and phase 2 detoxification. Its main function, however, is the regulation of proteasomal subunit gene expression in response to ER-stress associated with a compromised proteasome (bounce back), and thereby regulating the proteostasis of cells. NGLY1 deficient cells have been found to lack a sufficient proteasome function. We thus believe 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.

Based on this biological link between NGLY1 and Nrf1 we are seeking for new therapeutic avenues to treat the symptoms associated with NGLY1 deficiency. To this end, we developed a patient-derived IPSC-screening system that compares the NGLY1-deficient cells to controls in which the mutation has been corrected in either one or both alleles with help of the CRISPRi-technology. We are screening a number of compounds in order to identify hits that are able to rescue the altered growth phenotype of the NGLY1-deficient cells and thus, compensate for the insufficient proteasome function.

(105) Isolation and Characterization of a Novel Lamprey VLRB Specific for Galactose-3-Sulfate on Glycoproteins and Glycolipids

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Glycans are known to undergo reversible modifications including methylation, acetylation, phosphorylation, and

sulfation. However, the expression patterns and biological functions of most glycan modifications are unclear. A major reason for this lack of knowledge is the paucity of detection reagents available to discern the spatial and temporal expression profile of these modifications. Using the sea lamprey as a model system, we have identified a variable lymphocyte receptor (O6) from a yeast surface display library generated from immunization with human type O erythrocytes, that specifically recognizes the terminal (3 S)Galβ1-4GlcNAc motif.Through a variety of glycan array methodologies and biophysical experiments, including isothermal calorimetry and co-crystallization, we have defined the specific epitope for this VLR. Screening of O6 on a glycoprotein microarray revealed a number of glycoproteins not previously known to carry galactose-3-sulfate such as bovine fetuin and human serum glycoproteins including transferrin.We demonstrated the utility of this VLR for the characterization of glycan sulfation on a variety of platforms, including glycoprotein array, western blotting, TLC overlay, flow cytometry and Immunohistochemistry. O6 will thus serve as a valuable reagent for future glycan structural analyses, glycomic analyses, and understanding the biological functions of galactose-3-sulfate in health and disease.

(106) Human Adenovirus type 5 increases host cell fucosylation and modifies LeY antigen expression

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The overexpression of specific host cell glycosyltransferases, including fucosyltransferases (FUTs), is characteristic of certain viral infections that alter the glycosylation of host cells. Infection with CMV (cytomegalovirus), HCV (hepatitis C virus), HSV-1 (herpes simplex virus type-1) and VZV (varicella-zozter virus) increase the expression of fucosylated epitopes, including antigens sLe^x (Siaα2-3Galβ1-4(Fucα1-3) GlcNAcβ1-R) and Le^y (Fucα1- 2Galβ1-4(Fucα1-3) GlcNAcβ1-R). In this work, we aimed to establish whether infection with human adenovirus type 5 (HAd5), a well-known viral vector and infectious agent, causes changes in the glycosylation profile of the A549 cells, used as a model of lung epithelium, a natural target of HAd5. We demonstrate for the first time that HAd5 infection causes a significant increase in the cell surface *de novo* fucosylation, as assessed by metabolic labeling, and that such modification is dependent on the expression of viral genes. The main type of increased fucosylation was determined to be in α1-2 linkage, as assessed by UEA-I lectin binding and supported by the overexpression of FUT1 and FUT2. Also, HAd5-infected cells showed a heterogeneous change in the expression profile of the bi-fucosylated Le^y antigen, an antigen associated with enhanced cell proliferation and inhibition of apoptosis. We suggest, that in order to create a favorable cellular environment for viral infection or replication, HAd5 increases host-cell fucosylation and modifies Le^y antigen expression.

(107) Development and Standardization of Assays to Measure Plasma and Sera Glycosidase Activities in Normal and Disease Physiology

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Glycosidases are normally present in the bloodstream of healthy mammals and reflect, at least in part, a recently discovered intrinsic mechanism of secreted protein aging and turnover. This mechanism includes the sequential and temporal hydrolysis of terminal N-glycan linkages resulting in the exposure of cryptic ligands of endocytic lectins. Such lectins of various and distinct binding specificities are engaged in the recognition and turnover of these aged secreted glycoproteins. Recent studies have demonstrated that the expression of endogenous glycosidases are altered during diseases such as sepsis and colitis, and that the resulting change in glycoprotein clearance and abundance was in fact the cause of disease. We have set-up, developed, and optimized assays of neuraminidase (Neu), β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase, and α-fucosidase activities measured at both lysosomal and blood pH to obtain reproducible measurements of basal levels in order to subsequently detect glycosidase changes in diseases including mouse and human sepsis. Each assay is over 95% inhibited by competitive binding of saccharide antagonists and each assay is able to detect and measure specific activities at both lysosomal pH and blood pH. Profiles of normal mouse and human glycosidase activities in the blood plasma and sera have been completed and will be presented. In subsequent studies now ongoing, laboratory mice serving as experimental models have received comparable infections by different bacterial pathogens known to cause sepsis in humans. Multiple timepoints post-infection are analyzed for changes in glycosidase activities during sepsis. Recent data involving neuraminidase activity measurements have revealed alterations that are different in Gram-positive and Gram-negative sepsis, and which further cause disease. Ongoing analyses include comprehensive measurements of neuraminidase, β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase, and α-fucosidase activities to obtain activity profiles in both mouse and human sepsis caused by different pathogens.

(108) Glycan Microarray and Glycomics Services through the National Center for Functional Glycomics, the Harvard Medical School Center for Glycosciences, and the BIDMC Glycomics Core

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The National Center for Functional Glycomics (NCFG) at BIDMC at Harvard Medical School (HMS) is one of four Glycomics-related Biomedical Technology Resource Centers in the U.S., funded by the National Institutes of Health, NIGMS. The NCFG is an outgrowth of the successful Consortium for Functional Glycomics (CFG) glycan microarray resources. The overall goal of the NCFG is to "provide innovative leadership in developing technologies for studying the functions of cellular glycomes and for glycomic analyses, and understand the *protein-glycan interactome*." Our current research initiatives center on 3 Technology Research & Development Projects that revolve around expansion of glycan microarray technologies, development of "Shotgun Glycomics" as a general method for studying natural cell-derived glycan recognition, and development of different glycan display technologies, such as bead-displayed glycans for flow cytometry. Our efforts have expanded to include antibodies produced in lamprey (VLRBs) as *detectors* and *disruptors* of glycans and their interactions, heterogeneous glycopeptide libraries based on naturally occurring presentations, and other types of bead platforms as discovery tools. The paramount service offered through the NCFG is the analysis of glycan binding proteins (GBPs) on our collection of glycan microarrays, as fee-for-service and through collaborative research. The CFG mammalian glycan array, with ~600 glycans, is available through requests to the CFG Steering Committee, as are Microbial glycan microarrays. Additionally, we offer a multitude of defined glycan arrays, including mannose-6-phosphate compounds and glycopeptides. The number and diversity of glycan arrays is continuously growing, and now includes glycans generated by companies supported by NCI/SBIR/STTR grants. An exciting development within the NCFG has been shotgun glycan microarrays, containing the undefined mixture of glycans isolated from a tissue, cell, organism, or fluid. The libraries of glycans are printed as arrays for analysis with GBPs, and bound glycans are targeted for further characterization. New shotgun glycan libraries are continually being generated, and requests for shotgun microarrays of new materials can be directed to the NCFG. Our Center also offers comprehensive Glycomics analyses using MALDI-TOF and LC-MS mass spectrometry platforms. We routinely perform glycan profiling (glycolipids, N- and O-glycans) as well as sialic acid linkage analyses on all type of biological samples including serum/plasma, purified glycoproteins, cultured cells and tissue samples. We focus on technology development in the glycosciences and defining cellular glycomes important in human biology and disease, and we aim to support the community in their endeavors into defining protein-glycan interactions. http://ncfg.hms.harvard.edu/home *P41GM103694*

(109) Differential regulation of mammalian neuraminidases by Tlr4 in Gram-negative sepsis

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Age related remodeling of N-glycans in the bloodstream has been identified as a mechanism determining glycoprotein half-lives and clearance via endocytic lectin receptors including the hepatic Ashwell-Morell receptor (AMR). In a mouse model of lethal Gram-negative sepsis caused by infection with the human bacterial pathogen *Salmonella enterica* serovar Typhimurium (ST), we measured an increased rate of N-glycan remodeling by de-sialylation among blood glycoproteins with ensuing AMR ligand formation, resulting in rapid decreases in the abundance and function of two host anti-inflammatory enzymes, tissue non-specific and intestinal alkaline phosphatase (TNAP and IAP). The accelerated aging of TNAP and IAP by an increased rate of de-sialyation with the subsequent AMR-dependent clearance of TNAP and IAP bearing reduced half-lives in sepsis was due to the induction of host neuraminidase (NA) activity in blood. The ST pathogen does not encode a neuraminidase. Neu induction includes Neu1 and Neu3 isoforms and can be recapitulated by isolated lipopolysaccharide (LPS) endotoxin present among Gram-negative bacteria. To address whether LPS invariably induces host Neu expression, we also studied sepsis caused by *Salmonella enterica* serovar *Choleraesuis* (SC), a Gram-negative hypervirulent variant that we have characterized and published, and which exhibits a 100-fold reduced oral LD_{50} in WT mice. The hypervirulence phenotype is accompanied by elevated serum inflammatory cytokine levels and increased bacterial burdens in spleen and liver tissues relative to conventionally-virulent ST. We tested the hypothesis that LPS structural and functional differences between these two closely-related *Salmonella* strains may contribute to the mechanistic basis of hypervirulence. Since the physiological recognition of LPS occurs via Toll-like receptor 4 (Tlr4), we measured the ability of SC to infect *Tlr4*$^{-/-}$ mice and observed a 20- to 100-fold increase in oral LD_{50} relative to infection of WT mice, an increase in host protection similar to that observed following ST infection. Surprisingly, SC failed to induce host Neu activity and

expression, and the infected animals maintained normal AP levels prior to death. These findings suggest alternate host pathways that include Tlr4 function and differentially involve Neu induction in the pathogenesis of sepsis comparing Gram-negative ST and SC bacterial pathogens.

(110) Genotype-Phenotype Correlations for Protein O-Linked Mannose N-Acetylglucosaminyltransferase 1 (POMGNT1) in Congenital Muscular Dystrophy

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Congenital muscular dystrophy (CMD) is a heterogeneous family of inherited muscle disorders. A subtype of CMD known as dystroglycanopathy is classified by hypoglycosylation of alpha-dystroglycan (α-DG) that arises from defects in the protein O-mannosylation biosynthetic pathway. One enzyme involved in the O-mannosylation pathway is protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase (POMGNT1). POMGNT1 catalyzes addition of N-acetyl-D-glucosamine to an O-mannose structure in a β1,2-linkage. Mutations in the gene encoding POMGNT1 have been observed in patients with various dystroglycanopathies ranging from mild to severe disease. Our work examines the role of 13 point mutations in POMGNT1 in an attempt to define a genotype-phenotype correlation. The effects of the selected mutations on enzyme characteristics are not well established. Therefore, we sought to identify mutation-derived changes in enzyme kinetics and stability. Preliminary data indicates that some of the variant proteins were not capable of being stably expressed in a mammalian cell line. Kinetic analysis uncovered both impaired and catalytically dead variants of the enzyme. A thermal shift assay revealed that all variants able to be expressed to date exhibited similar stability parameters. We are currently working to characterize all variants including rescuing POMGNT1 knockout cell lines for IIH6 reactivity, laminin binding, and Lassa pseudovirus entry. Understanding genotype-phenotype correlations in this glycosyltransferase could facilitate the design of personalized approaches for therapy. This work was supported in part from a grant from NIH/NIGMS (LW and DL, R01GM111939).

(111) Galectin-4 interaction with CD14 triggers the differentiation of monocytes into macrophage-like cells via MAPK signaling pathway

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Galectin-4 (Gal-4) is a β-galactoside-binding protein specifically expressed in the gastrointestinal (GI) tract. Although intensive studies have been done for the function of other isoforms of galectins, the immune-regulatory function of Gal-4 in environments such as intestinal mucosa remains ambiguous. Here, we demonstrated that Gal-4 specifically binds to CD14 on monocytes and induces the differentiation of monocytes into macrophage-like cells through MAPK signaling pathway. Gal-4 induced phenotypic changes of monocytes by altering the expression of various surface molecules on monocytes such as chemokine receptors, toll-like receptors (TLRs), co-stimulatory molecules and HLA-DR. Furthermore, the functions of monocytes were affected by Gal-4. While the cytokine production and matrix metalloproteinase (MMP) expression were increased in Gal-4-treated monocytes, their phagocytic capacity was reduced. Concomitant with these changes, Gal-4-treated monocytes became adherent and elongated with a *spindle*-like morphology, and also showed an increased expression of macrophage markers. Notably, we found that Gal-4 interacts with CD14 and activates MyD88/MAPK signaling cascade (p38 and c-Jun). The effect of Gal-4 on monocytes was completely abrogated by anti-CD14Ab. Also, Gal-4 could not increase the macrophage marker expression in MyD88-deficient monocytes. Therefore, these findings suggest that Gal-4 may regulate the immune responses through the activation and differentiation of monocytes.

(112) Role of sialyl 6-sulfo Lewis X in anti-tumor immunity against oral squamous cell carcinoma

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Tumor-infiltrating lymphocytes (TILs) reportedly play a pivotal role in antitumor immunity against oral squamous cell carcinoma (OSCC); however, mechanisms governing TIL recruitment to OSCC tissues remain to be clarified. This study was undertaken to assess a potential association between TILs and high endothelial venule (HEV)-like vessels that express sialyl 6-sulfo Lewis X (LeX).OSCC tissue sections (n = 41) were subjected to immunohistochemistry for sialyl 6-sulfo LeX and CD34 to allow quantitation of HEV-like vessels. Triple immunohistochemistry for sialyl 6-sulfo LeX and either CD3 and CD20 or CD4 and CD8 was conducted to determine which lymphocyte subset is more closely associated with HEV-like vessels. HEV-like vessels expressing sialyl 6-sulfo LeX were detected in 27 of 41 (65.9%) OSCC cases, and these vessels were more frequently found

in early disease (T1/T2 stages) compared with advanced (T3/T4) stages. The number of T cells attached to the inner surface of these HEV-like vessels was significantly greater than that of B cells, while the number of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells did not differ significantly. Interestingly, sialyl 6-sulfo LeX was also expressed on the membrane of a fraction of OSCC cells, and $CD8^+$ cytotoxic T cells were almost exclusively found attached to these carcinoma cells. Sialyl 6-sulfo LeX is displayed not only on HEV-like vessels but also on OSCC cells and may potentially function in antitumor immunity against OSCC.

(113) Role of the ST6Gal-I sialyltransferase in protecting tumor cells from hypoxia

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A change in surface glycosylation was one of the first identified hallmarks of a cancer cell, however, the functional effects of altered glycosylation on cancer cell phenotype remain poorly-understood. Our group and others have shown that ST6Gal-I, a sialyltransferase that adds α2-6-linked sialic acids to *N*-glycosylated proteins, is upregulated in many cancer types including ovarian and pancreatic cancer. ST6Gal-I acts as a pro-survival factor in a variety of settings, including resistance to chemotherapeutic drugs. In the current study, we describe a novel function for ST6Gal-I in promoting tumor cell survival within the hypoxic tumor microenvironment. Hypoxia leads to the induction of hypoxia inducible factors (HIFs), which are transcriptional regulators of the cellular response to hypoxia. HIFs, such as HIF-1α, direct the transcription of many proteins necessary for cells to survive under low oxygen tension. Using ovarian and pancreatic cancer cells with ST6Gal-I overexpression or knockdown, we recently determined that ST6Gal-I activity promotes the accumulation of HIF-1α. This augmented accumulation is accompanied by increased transcription of HIF-1α target genes including the glucose transporters, GLUT1 and GLUT3, and the glycolytic enzyme, PDHK1. These data suggest that cancer cells with high ST6Gal-I expression are better adapted to survive within a hypoxic microenvironment. We further observed that both the AMPK and NFκB pathways have increased activation in cells with high ST6Gal-I expression subsequent to culture in hypoxia. Additionally, long-term culture in hypoxia selects for cells with high ST6Gal-I expression. Finally, we show that ovarian cancer cells with forced overexpression of ST6Gal-I injected into the peritoneum, a highly hypoxic microenvironment, have increased capacity to disseminate and form secondary tumors. Taken together, these data highlight a novel glycosylation dependent mechanism that aids cancer cells in surviving hypoxic microenvironments, thereby fueling tumor progression and metastasis.

(115) HS S-domains that accumulate in ATTR amyloidosis patients accelerate and mediate formation and cytotoxicity of transthyretin fibrils

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Introduction: Heparan sulfate (HS) is a sulfated linear polysaccharide composed of a repeating disaccharide of glucosamine and hexuronic acid. The structure of HS is heterogeneous. The highly sulfated domains of HS, referred to as HS S-domains, are made up of repeated trisulfated disaccharide units [iduronic acid (2 S)-glucosamine (NS, 6 S)–] and are selectively remodeled by Sulfs, extracellular endoglucosamine 6-sulfatases. HS S-domains are critical for molecular interactions between HS and its ligands, and for signal transduction of several growth factors. Although HS has been implicated in the pathogenesis of various amyloidoses including ATTR amyloidosis, pathological roles of HS S-domains in amyloidoses remain to be elucidated. Here, we investigated the roles of HS S-domains in the formation and cytotoxicity of transthyretin (TTR) fibrils.

Materials & methods: Paraffin-embedded sections of ATTR amyloidosis kidneys were stained with the RB4CD12 anti-HS S-domain antibody. The wild-type and V30M mutated TTR 12-35 fragments, and the 105-123 fragment were purchased from Eurofins Genomics (Tokyo, Japan). The TTR 81-127 fragment was expressed in *Escherichia coli* and purified. Heparin, a structural analogue of HS S-domains, was conjugated with bovine serum albumin (BSA). Fibril formation of TTR fragments was monitored in plates that were coated with BSA or heparin-BSA conjugates by using thioflavin T. In order to assess the role of HS S-domains, the HS S-domains within the heparin-BSA conjugates were eliminated by treating wells of heparin-BSA-coated plates with culture medium of Chinese hamster ovary (CHO) cells that stably express human Sulf-2 (CHO-HSulf-2). TTR fibrils were obtained by incubating TTR fragments (10 μM) at 37 °C for 5 days and labeled with a fluorescent dye. CHO and CHO-HSulf-2 cells were incubated with TTR fibrils at 37 °C for 12 h, after which percentages of fibril-positive cells were determined by using a

confocal microscope. Cytotoxicity of TTR fibrils was analyzed by using a mitochondrial membrane potential-sensitive fluorophore, tetramethylrhodamine ethyl ester.

Results & Conclusions: HS S-domains accumulated in the kidney of an ATTR amyloidosis patient. Heparin accelerated the fibril formation of TTR fragments in an HS S-domain-dependent manner. TTR fibrils interacted with cells via HS S-domains at the cell surface, and the cytotoxicity of TTR fibrils was mediated by the HS S-domains. Our findings unveil the pathological roles of HS S-domains in ATTR amyloidosis. We also propose that the enzymatic remodeling of HS S-domains by Sulfs is a novel strategy for targeting formation and cytotoxicity of amyloid fibrils.

(116) Identifying C-mannosylated Proteins in RAW264.7 Cells

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C-mannosylation is a specific type of glycosylation in which α-mannose is covalently bound to the C_2 atom of a tryptophan via a carbon-carbon bond. Presently, it is known to occur on proteins that contain the W-x-x-W-x-x-W/C motif found within the Thrombospondin Type-1 Repeat (TSR), with 'x' denoting any other amino acid. *C*-mannosylation has been implicated in a few cellular functions, including protein secretion and type I cytokine receptor function. Previous research has shown that exogenous *C*-mannosylated peptides derived from TSR containing proteins upregulate the production of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNF-α) in RAW264.7 macrophage cells. This upregulation is substantially increased in the presence of Hsc70. When these peptides are internalized y RAW264.7 cells, a binding interaction occurs between the two proteins, which also correlates with increased TNF-α levels. With the knowledge that exogenous *C*-mannosylated peptides and Hsc70 can influence cytokine production, this study has focused on identifying endogenous *C*-mannosylated proteins in RAW264.7 cells that interact with Hsc70. The potential of a naturally occurring interaction between these two proteins could elucidate the importance of *C*-mannosylation in the innate immune response. Through a Hsc70 co-immunoprecipitation, a potentially *C*-mannosylated protein has been identified in Junctophilin-1 (JPH1). JPH1 forms a junctional membrane complex (JMC) between the endoplasmic reticulum and plasma membrane of excitable cells, facilitating communication between calcium ion release channels. When comparing the nano-LC MS/MS results for JPH1 against a mouse database, 69 separate PSMs were shown where JPH1 carries a hexose. MS/MS data suggests that the hexose appears on a single tryptophan in a peptide bearing the sequence: E-G-E-W-A-N-N-K. This indicates the potential for JPH1 to be an example of non-canonical *C*-mannosylation. This work was supported by NSF grant OIA-1738707.

(117) A Role for O-Fucosylation in Notch folding and trafficking

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Notch is a transmembrane protein receptor that mediates direct cell-cell communication at the cell surface. Mouse NOTCH1 is *O*-fucosylated by Protein *O*-fucosyltransferase 1 (POFUT1) at predicted consensus sequences ($C^2XXXX(S/T)C^3$) found within 17 of the 36 total Epidermal Growth Factor-like (EGF) repeats in its extracellular domain. Recent reports have demonstrated that *O*-fucose residues on EGF8 and 12 are directly involved in intermolecular interactions with Notch ligands and play a crucial role in Notch-ligand binding. Additional studies have shown that *O*-fucose residues on EGF repeats are also involved in intramolecular interactions with underlying amino acids in the EGF repeat, stabilizing their structure. Since *O*-fucosylation occurs in the endoplasmic reticulum (ER), and POFUT1 only modifies EGF repeats after they are folded, these results are consistent with reports that addition of *O*-fucose residues are essential for efficient Notch trafficking from the ER to the cell membrane. Nonetheless, other studies suggest addition of *O*-fucose is not required for folding and trafficking of Notch. For instance, POFUT1 has been reported to have a fucosyltransferase-independent chaperone activity, and other reports suggest that the trafficking defects are not seen in all cell systems. To further examine the role of *O*-fucose modifications in Notch trafficking, we have tested whether mutant forms of POFUT1, known to have significantly reduced enzymatic activity, are able to rescue addition of *O*-fucose to NOTCH1 EGF repeats in *POFUT1*-null HEK293T cells, and whether these same mutants can rescue NOTCH1 localization defects in the same cells. The POFUT1 mutations tested were from patients with *Dowling-Degos*Disease (DDD: R240C, M262T, S356F, and R366W), a recently-described patient with severe developmental defects (S162L), and a mutation in the active site which was used in many studies proposing that POFUT1 has fucosyltransferase-independent chaperone activity (R240A). Interestingly, all POFUT1 mutants, including R240A, were able to add *O*-fucose to EGF repeats in cells to some extent except for S356F. In addition, all could at least partially rescue NOTCH1 trafficking in *POFUT1*-null cells, except for S356F. These data strongly suggest that addition of *O*-fucose to NOTCH1 is essential for efficient trafficking. Supported by NIH grant GM061126.

(118) Bacterial Polysaccharides in Cancer Therapy, 1866-2018

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Historical: From 1866, to 1892, German and French oncologists observed that sarcoma patients with **accidental erysipelas** exhibited tumor regression and ablation[1-3]. These physician-scientists deliberately infected patients with the erysipelas causing organism found by Fehleisen[2] to be *Streptococcus erysipelatos*, now called *S. pyogenes*, with remarkable success in both humans[1-3] and spontaneous tumors in dogs. [1]Busch W (1868) In: 'Verhandlungen artzlicher Gesellschaften', *Berl. Klin. Wochenschr. 5:* 137-138. [2]Fehleisen F (1882) Uber die Zuchtung der Erysipelkokken auf kunstlichem Nahrboden und die Ubertragbarkeit auf den Menschen. *Dtsch. Med. Wochrnschr. 8:* 553-554. [3]Spronck CHH (1892) Tumeurs malignes et maladies infecticuses. *Ann. Inst. Pasteur* 6: 683-707. Erysipelas is a skin rash, caused by beta-hemolytic group A *Streptococcus pyogenes* bacteria. Incidental Erysipelas infections in cancer patients caused tumor ablation (1866-1920).

• COLEY'S TOXIN: Independently, William B. Coley[4-8], a surgeon at Cornell University found a 7 year old case in the hospital records showing a sarcoma regression with an accidental erysipelas infection. He independently injected *S. erysipelatos* in cancer patients, with remarkable success. Coley heat-killed the cultures, filtered out the bacteria and found the same tumor hemorrhagic results from culture medium. Roger, in France reported that co-culture of *Bacillus prodigiosus (now Serratia marcescens)* had an adjuvant effect in producing *S. erysipelatos* antibodies in rabbits. Coley, co-cultured these organisms, obtaining more potent antitumor preparations "**Coley's Toxin**". (Coley reviewed 20 years of work in this area in 1910). Pfizer produced Coleys Toxin for 20 years. A large number of reports supported the success of this approach. Beebe and Tracy, at a New York Cancer Research Center, treated 5 dogs with transplantable sarcomas with Coley's Toxin with 5 cures.

• Shear, *et al.*, 1943, characterized the tumor hemorrhagic principle in Serratia as a polysaccharide fraction, no peptide, with lipid and phosphate, hexosamine, hexose, methyl pentose. Hellerqvist, *et al.*, in the 1990's, independently found the active principle from Group B Streptococcus (GBS) causing "early onset disease" neonate lung destruction was a 270,000da polysaccharide with lipid, phosphate, containing hexosamine, hexose and methylpentose. They applied it to a mouse tumor model, surmising that tumors attracted the same kinds of capillary, Hellerqvist's group conducted a Phase I clinical trial with 33% effectivity in stage 4 patients: Hellerqvist's group isolated the receptor on hypoxic driven capillaries as SP55 in all animals and HP59 in humans, later shown to be Sialin SLC17A5. With additional new data, it appears that these two polysaccharides, one from a gram negative Serratia and one from a gram positive GBS may have the same receptor and may cause the same effect, complement activation specifically on tumor capillaries.

(119) IgE glycosylation patterns in human disease

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IgE antibodies have an indisputable role in allergic diseases which affect a staggering 30-40% of the world's population. IgE is the least common antibody class in the circulation. These are the most heavily glycosylated monomeric antibodieswith seven N-linked glycosylation sites distributed across each heavy chain. In addition to allergy, a host of other diseases implicate IgE in pathogenesis, although mechanisms are not well understood. These include immune deficiencies, systemic lupus erythematosus, and helminth infections. However, there is currently no criteria to define what makes IgE pathogenic. Indeed, approximately one half of individuals with food-specific IgE do not experience allergic symptoms upon ingestion of the relevant food. This suggests antigen specificity alone does not determine IgE biologic effects. IgG antibodies are the most common class in the circulation and are perhaps the best studied. The role of the singleFc glycan in IgG biology has been well established. Regulation of IgG glycosylation has been described across several clinical conditions including infection, pregnancy, autoimmune disease, and vaccination. A number of groups, including ours, have analyzed the glycosylation of IgE. However, this has been limited to non-allergic samples, including recombinant IgE, hyper IgE, and myeloma IgE. Here, we examined the glycosylation of primary IgE from allergic samples to define glycosylation patterns of pathogenic IgE. This initial work will serve as a foundation to understand how IgE glycan configurations are regulated during homeostasis and disease.

(120) Galectin 8 in Trypanosoma cruzi-induced myocarditis

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In Chagas disease, the American trypanosomiasis, it is known that cardiomyopathy is based on the induction of a strong inflammation due to *Trypanosoma cruzi* persistence in cardiac tissue. Gal-8, a Galectin widely distributed in different tissues that can works through an autocrine/paracrine way, is involved incellular adhesion, migration, apoptosis, etc. It has been associated either as a pro-inflammatory or anti-inflammatory molecule by different authors in several models. To

analyze the role of Gal-8 in an inflammatory infectious context, we used a murine chronic *T. cruzi* infection model. C57BL/6 J (B6, WT) and B6Gal-8 knock out (KO) mice were infected with the Ac strain (DTU TcI) *T. cruzi*. Four month post-infection, the cardiac tissue from KO mice showed increased inflammation score in comparison with WT ($p = 0.0119$). No differences were observed in fibrosis degree and tissue parasite levels. To identify the immune population in the inflamed cardiac tissue, a flow-cytometry analysis was performed. Similar values were found in the percentage of cardiac T lymphocytes (CD3+), and their different subpopulations, between KO and WT mice. We observed a rise in the frequency of macrophages ($p = 0.0021$) in KO heart tissue compared to WT, in agreement with increased cardiac CCL-2 levels. A strong increment in neutrophil numbers was observed in KO vs. WT ($p = 0.0097$) mice that, however, did not correlate with cardiac and systemic CXCL1 and CXCL2 levels. These results suggest that the neutrophil rise may be instead related to Gal-8 preaparesis induction mechanism. Taken together, our results suggest that Gal-8 participate as an anti-inflammatory molecule in *T. cruzi* chronic infection.

(121) Why do Fringe enzymes modify O-fucose on some Notch EGF repeats but not others?

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Notch (N) signaling receptors are involved in myriad developmental, cell differentiation, and cell regulatory events. Disruptions to N signaling result in embryonic lethality, developmental defects, cancers, and have been implicated in various other pathologies. Four N receptors exist in mammals (N1-N4) all of which are single-pass transmembrane proteins containing 29 to 36 tandem EGF-like repeats (EGFs) in their extracellular domains. Signaling through the N receptors involves ligands on adjacent cells interacting with the N extracellular domain, triggering proteolysis events that release the N intracellular domain to the nucleus. The ligand/receptor interactions are modulated *via* glycosylation on the receptor EGFs. These glycans are initiated by PROTEIN *O*-FUCOSYLTRANSFERASE 1 adding fucose to EGFs with a consensus sequence for fucosylation. A sub-set of fucosylated EGFs are then subject to elongation by a family of three Fringe beta-3-N-acetylglucosaminyl-transferases: LUNATIC, MANIC, and RADICAL FRINGE. Broadly speaking on N1, RADICAL FRINGE modifies a sub-set of EGFs modified by MANIC FRINGE, which in turn modifies a sub-set of EGFs modified by LUNATIC FRINGE, which modifies a sub-set of all fucosylated EGFs. Previous studies have additionally suggested that the primary amino-acid sequence of a single EGF is necessary, but may not be sufficient for directing Fringe specificity. Despite close sequence identity between corresponding EGFs on the N1 & N2 receptors, we now have data showing that in at least some instances, corresponding EGFs between these receptors possessing high sequence identity can exhibit dramatically different specificity as Fringe acceptor substrates. This suggests that position in the tandem sequence of EGFs in the extracellular domain of the N receptors could be contributing to Fringe acceptor substrate specificity. To address the question of how Fringe specificity for an EGF is determined, we have begun swapping corresponding EGFs with high sequence identity between the N1 and N2 receptors. We then analyze the degree of Fringe glycosylation on the swapped constructs compared to the native control sequences. Once we have mapped the effects of swapping individual EGFs, we can correlate the *in vivo* specificity with *in vitro* specificity. We can then iterate this process, swapping multiple EGFs, with the intent of fully mapping the acceptor substrate specificity determinants for Fringe enzymes with regard to N EGFs. This work is supported by NIH grant GM061126.

(122) ST6Gal-1 deficiency is associated with development of GVHD-like autoimmune symptoms, myeloid biased hematopoietic reconstitution post whole bone marrow transplant and reduced cycling of hematopoietic stem cells

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Graft vs host disease (GVHD) is an immunologically mediated disease and is one of the major complications arising in patients receiving hematopoietic stem cell transplants (HSCT). The incidence of GVHD is between 30- 60%, with a mortality rate of 50% thus significantly limiting the clinical application and benefits of HSCT as therapy for various hematological cancers. We report here preliminary observations wherein recipient C57/B6 mice deficient in ST6Gal1 specifically develop skin lesions post transplant with syngeneic wild type WBM. (from other C57/B6 mice). Apoptotic keratinocytes with lymphocyte infiltration were observed in histological analysis of skin punches, implicating early phase GVHD. All wild type recipients remain asymptomatic.

ST6Gal1 deficient recipient mice had more donor derived myeloid cells in peripheral blood post transplant. We observed further that the wild-type donor originated cells had reduced cell surface SNA reactivity while the recipient originated LSK and HSC that were natively ST6Gal1 deficient gained cell surface SNA reactivity. This suggests either redistribution of SNA-binding a2,6-sialyl glycans between donor and recipient cells, or extrinsic sialylation by extracellular ST6Gal-1 is a major mechanism in maintaining a2,6-sialylation of early hematopoietic progenitors.

The absence of systemic ST6Gal-1 may be a driver in the observed GVHD- like symptoms as well as the myeloid bias in ST6Gal-1 deficient animals, despite receiving wild-type marrow transplants. We further observed increased numbers

of hematopoietic stem cells in ST6Gal1 deficient mice in the quiescent G0 phase at baseline, exhibiting reduced ability to cycle upon induction with poly I:C.

Taken together, our data point to the ST6Gal1 status as important in bone marrow transplantation conditions, affecting the ability of stem cells to respond to situations demanding hematopoietic output as well as the propensity in the development of complex autoimmune conditions. (This work was supported by the Program of Excellence in Glycosciences P01-HL107146)

(123) DANGO: An MS data annotation software for Glycolipidomics

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We are developing MS data annotation software for glycosphingolipid analysis, named DANGO (Data ANnotation system for GlycolipidOmics). Glycosphingolipids (GSLs) are comprised of glycans attached to a ceramide lipid moiety and are not just structural elements of cells but also participate in intra- and extracellular signaling. The heterogeneity and structural diversity of GSL glycan headgroups and ceramide backbones are modulated by metabolic, environmental, and pathologic conditions and also contribute to selective biochemical functions. Ceramide heterogeneity in GSLs arises from the structural variation of their fatty acid and sphingosine components, including the degree of hydroxylation, unsaturation, and alkyl chain lengths. Therefore, we have developed an annotation system using structural databases for GSL glycan moieties as well as ceramide compositions which can be optimized to meet individual user requirements.

DANGO is as an extension (plugin) to GRITS Toolbox (http://www.grits-toolbox.org) which is freely available software for processing and archiving glycomics data. The software also includes an integrated annotation module, GELATO, for annotating glycomics data. Implementing DANGO as a plugin to the GRITS Toolbox allowed us to repurpose the GELATO functionality for the glycan portion of the GSL. It also allowed the development of an extensive set of investigation and visualization widgets for GSL.

DANGO supports processing and annotation of high throughput MS data analysis, thereby significantly reducing the time required for data interpretation compared to manual data interpretation. Using data generated with our improved MS workflow, which sequentially profiles GSL glycan and ceramide moieties, DANGO is able to annotate three hundred MSn spectra against a million candidate GSL structures within a few minutes. Annotation results are displayed as tabular representations or as annotated MS spectra. A merge report tool is available for the comparison of multiple annotation results side-by-side in a single display. We also created additional tools complementary to DANGO for supporting annotation and analysis. One of these tools is a database generator for the lipid portion of the GSL allowing the generation of customized GSL databases based on user specifications. A similar system for the creation of custom glycan structure databases is available as well.

We will present the latest version of the DANGO software and are seeking collaborative partners interested in applying our software to their biological questions, thereby helping us to improve the system and make it more user friendly.

(124) A Well-Characterized Human Chimeric Anti-Tn Monoclonal Antibody as a Tumor Diagnostic Biomarker

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There is a need to identify tumor-associated biomarkers for diagnostics and therapy. Among several tumor biomarkers under consideration is the Tn antigen, which is an abnormal mucin type O-glycan (GalNAc-a1-O-Ser/Thr) expressed on glycoproteins in a majority of carcinomas. Expression levels correlate with metastasis and poor survival, and can 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 was generated in a murine IgM framework and termed Re-BaGs6, while the recombinant human chimeric anti-Tn antibody was generated using a human IgG1 framework and termed Remab6. Neither of these antibodies exhibit cross reactivity with potentially cross-reactive glycans containing terminal GalNAc, such as blood group A and Forssman antigens. 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. Using Remab6 in immunoprecipitation experiments with a colon cancer cell line followed by LC-MS/MS on the Orbitrap Fusion Lumos, allowed us to identify several Tn containing glycoproteins that will be useful in analyses as tumor biomarkers.

Immunohistochemistry clearly distinguished staining in Tn+ tissues in IEC-*Cosmc* knockout mice and human cancer cell block sections. We have also used the recombinant anti-Tn antibodies to screen a large panel of human cancer tissues in array format. 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.]

(125) A missense mutation in SLC39A8, a manganese transporter linked to schizophrenia, is associated with specific changes in plasma N-glycosylation

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Schizophrenia is a chronic and severe mental illness with strong heritability. Recent Genome Wide Association Studies (GWAS) identified a strong association between the disorder and a missense mutation (A391T) in the zinc/manganese transporter SLC39A8 ($p< 8 \times 10^{-15}$). Manganese (Mn) is a critical cofactor for many enzymes including certain glycosyltransferases; enzymes that attach sugar molecules to proteins and lipids to regulate their function. Patients with severe mutations in SLC39A8 (not A391T) present with symptoms of a type II congenital disorder of glycosylation, and treatment with Mn or precursors of galactosylation reverses deficient transferrin glycosylation and some clinical phenotypes. The purpose of our study is to determine the link between the SLC39A8 A391T missense mutation linked to schizophrenia and the glycosylation pathway. Using the Partners Biobank, a biorepository of samples linked to the electronic medical record and genomic data, we optimized a search algorithm with 65% accuracy to identify patients with schizophrenia. After confirming the diagnosis via chart review, controls and patients with schizophrenia were stratified into groups based on SLC39A8 genotype, and samples of serum and plasma were obtained from the Biobank for study. We confirm that the A391T mutation results in decreased serum manganese with no effect on zinc.Analysis of plasma N-glycans shows a reduction in high molecular weight structures by ~25% in A391T mutation carriers, suggesting reduced activity the Mn-dependent enzyme β(1,4)-galactosyltransferase. Analysis of plasma N-glycans from patients with schizophrenia independent of SLC39A8 genotype identified a 20% increase in fucosylation and 30% reduction of sialylation relative to controls, though these modifications can also be affected by inflammation, environmental exposures, and medications that may be present in the disease group. Investigation of a subset of individuals with schizophrenia and the A391T mutation are ongoing. In summary, we demonstrate that plasma glycosylation is altered in individuals harboring the SLC39A8 A391T mutation, and hypothesize that similar changes in the developing brain result in the increased risk of developing schizophrenia in mutation carriers.

(126) Soluble glycosylated protein released from glycogen by rhGAA

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In studies on the degradation of glycogen by rhGAA a glycosylated protein core material glycogen was found which consists of about 5-6% of the total. However there was a question of why the glucose released by rhGAA still does not equal the amount of starting glycogen. Approximately 25% of the glycogen was unaccounted for based on glucose released. After incubation of glycogen with rhGAA until no more glucose is released no carbohydrate was detected from the incubation medium that elutes after glucose by HPAEC-PAD. If the incubation medium is boiled in 0.1 N HCl first, several oligosaccharides are then detectable. Incubation with trypsin or chymotrypsin also exposes oligosaccharides for detection. This appears to be a case of protein masking carbohydrate. The material in the medium binds to Dowex 50 W which is evidence of a charge as a protein and it is not bound by Concanavalin A. Glycogen does bind Concanavalin A. Given its characteristics the presence of this material in serum was suspected. It is present in serum either as an HCl extract or in a trypsin or chymotrypsin digest. The serum material was characterized the same as the material from the incubation medium. One oligosaccharide, which can not be degraded further by rhGAA, from the incubation medium and serum co-elute. These oligosaccharides contain *m*-inositol, *e*-inositol, sorbitol and glucose with two also containing xylose. This is is taken as an indication of a fraction of glycogen being degraded outside the lysosome. These results are taken as evidence that a portion of lysosomal glycogen degradation occurs outside the lysosome and outside of the cell. This now raises the question of where this material may be degraded and if it is related to the residual glycogen in the Pompe mouse tissue following ERT. (Supported by Genzyme GZ-2017-11679)

(127) Modeling organelle-specific O-glycosylation in driving liver tumor growth, invasion and metastasis

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Cancer progression is driven by the ability of tumor cells to invade surrounding tissues. Invasiveness correlates with perturbation of a covalent modification of cell surface proteins: O-glycosylation. However, the molecular mechanisms are still unclear. Here, we show that O-glycosylation initiating enzymes are activated during liver tumor growth by intracellular relocation from Golgi to ER. In a Nras/shp53 mouse liver cancer model, co-expressing an ER-targeted glycosyltransferase GALNT1 (ER-G1) massively increased tissue growth, invasion and metastases. ER-G1, but not its Golgi-localized counterpart, strongly stimulates glycosylation of the key cancer matrix metalloprotease MT1-MMP, this modification being essential for collagenase activity. Conversely, expression of an ER-targeted inhibitor of GALNTs inhibits liver tumor progression in Nras/shp53 injected mice. In summary, our model comprehensively demonstrates the generation and analyses of the GALNTs Activation pathwaydriving liver tumor invasiveness; thus further characterization of glycosylation substrates will unveil mechanisms of tumor growth and help identify potential therapeutic targets in human liver cancer.

(128) The Development and Characterization of EGF Centric O-Fucose Antibodies

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The NOTCH1 protein is regulated at numerous levels, and glycosylation of the extracellular domain has emerged as a critical regulator *in vivo*. O-Fucosylation, one form of Notch glycosylation, is essential for NOTCH1 function. O-Fucose monosaccharides are added to Epidermal Growth Factor-like (EGF) repeats in the Notch extracellular domain by protein O-fucosyltransferase 1 (POFUT1) and can be elongated by the addition of N-acetylglucosamine (GlcNAc), a reaction catalyzed by the Fringe family of beta3-N-acetylglucosaminyltransferases. Studies suggest that fringe-deficiency, which leads to truncation of the O-fucose structure to a fucose monosaccharide, is associated with certain types of aggressive cancers. Therefore, development of reagents that can detect monofucosylated Notch1 protein could lead to new tools to detect and treat cancer.

We have developed a series of high-affinity EGF-specific O-Fucose polyclonal and monoclonal antibodies. These EGF centric antibodies require a more elaborate characterization scheme than other antibodies as they need to demonstrate both a preference for the O-Fucosylated species over the native peptide sequence and a specificity to the amino acid sequence. We use a multistep evaluation process to characterize our antibodies. 1) ELISA assay, to determine the preference of the antibody to the O-Fucosylated peptide over the unmodified form; 2) Cross-reactivity of the antibody to other peptide sequences modified with O-Fucose; and 3) Western Blot analysis with increasing wash stringencies; 4) Demonstration that Fringe modification abolishes reactivity. Using this process, we have authenticated our EGF centric O-Fucose antibodies and have demonstrated that they possess the preference and specificity needed for research into Notch signaling. The work was supported by R41 GM119879.

(129) Plasma glycomics predict cardiovascular disease in patients with ART-controlled HIV infections

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Even in the age of antiretroviral therapy, HIV$^+$ patients – including those who control disease well – are plagued by increased risk of secondary diseases. These sequelae, such as cardiovascular disease (CVD), are poorly understood and represent a major health burden. To date, predictive biomarkers of HIV-associated secondary disease have been difficult to identify and implement, limiting preventative care and management. We applied our adaptable and easy to deploy platform – multi-target and high-throughput glycomic analysis – to banked HIV$^+$ human plasma samples to identify potential glycan determinants that can act as prognostic biomarkers for CVD events. Using 324 patient samples, we identified a glycomic fingerprint that was predictive of future CVD events, but independent of various controlled variables, including CD4$^+$ cell counts, diabetes, age and birth sex, indicating that the plasma glycome may be a biomarker for HIV sequelae. This fingerprint constitutes an increase in stain with MAL-II, and a decrease in staining with Jac, RCA120, WGA, and ConA. These trends suggest that overall glycan staining is decreasing (ConA, Jacalin) and increasing in terminally sialylated residues (RCA-120, MAL-II). Our finding that glycans serve as novel biomarkers which classify HIV$^+$ patients with elevated risk for CVD reveals, in part, the untapped prognostic potential of the plasma glycome in human disease.

(130) Immunological responses against biodevices for humans

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Susceptibility to structural valve deterioration is one of the major drawbacks of bioprosthetic heart valves (BHVs). *N*-glycolylneuraminic acid (Neu5Gc) is an immunogenic dietary-carbohydrate antigen in humans because of inactivation of the gene encoding CMP-*N*-acetylneuraminic acid hydroxylase (*CMAH*), and all humans have circulating anti-Neu5Gc antibodies. We hypothesized that interaction of anti-Neu5Gc antibodies with Neu5Gc on BHVs could lead to immune response resulting in valve deterioration through calcification. We demonstrate Neu5Gc in both native calcified human valves as well as in calcified-BHVs, explanted from human patients, by HPLC and immunohistochemistry. Furthermore, anti-Neu5Gc IgGs were purified from native calcified human valves, validated by a glycan microarray. In the Neu5Gc-free *Cmah*-KO mouse model, anti-Neu5Gc antibodies promoted calcium deposits in subcutaneous implanted BHV discs, both with passive transfer of affinity-purified human anti-Neu5Gc IgGs, and by active-immunization of *Cmah*-KO mice with Neu5Gc-containing glyconanoparticles. Thus, co-existence of Neu5Gc/anti-Neu5Gc likely mediate BHV structural valve deterioration.

(131) Engineered Sialylation of Pathogenic Antibodies In Vivo Attenuates Autoimmune Disease

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Self-reactive IgGs contribute to the pathology of autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis. Paradoxically, IgGs are used to treat inflammatory diseases in the form of high-dose intravenous immunoglobulin (IVIG). Distinct glycoforms on the IgG crystallizable fragment (Fc) dictate these divergent functions. IgG anti-inflammatory activity is attributed to sialylation of the Fc glycan. We therefore sought to convert endogenous IgG to anti-inflammatory mediators in vivo by engineering solubilized glycosyltransferases that attach galactose or sialic acid. When both enzymes were administered in a prophylactic or therapeutic fashion, autoimmune inflammation was markedly attenuated in vivo. The enzymes worked through a similar pathway to IVIG, requiring DC-SIGN, STAT6 signaling, and FcgRIIB. Importantly, sialylation was highly specific to pathogenic IgG at the site of inflammation, driven by local platelet release of nucleotide-sugar donors. These results underscore the therapeutic potential of glycoengineering in vivo.

(132) Modulating Tn-Mediated Immune Suppression in Breast Cancer

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Cancers often develop rapidly due to downregulation of effector immune responses. Immune regulation can occur through cytokine secretion, signaling mechanisms, and/or checkpoint. Certain suppressor immune cells have been associated with all of these regulatory pathways and are thus implicated in tumor immune escape. Tumor cells have been shown to promote these suppressive mechanisms. This can occur through biochemical groups expressed on the surface of the cancer cell, which may signal to effector immune cells or putative immune suppressor cells. Some tumor-associated carbohydrate antigens (TACAs) are known to be associated with immune suppression in helminth-infected patients. One ligand for certain TACAs is CD301b (also known as MGL2), which is expressed on certain dendritic cell and macrophage populations of immune cells.

As TACAs and the CD301b ligand have been linked with immune suppression, cancer cells may use this signaling pathway as a mechanism to induce immune suppression. We hypothesize that $CD301b^+$ immune cells represent a suppressive immune cell population. We hypothesize that glycans expressed on the surface of breast cancer cells can stimulate activity and suppressive function of these $CD301b^+$ immune cells, leading to enrichment at the tumor site and inhibition of effector immune responses. Our objective is thus to inhibit this TACA-CD301b interaction, overcome immune suppression, and stimulate the anti-tumor immune response. Our specific aims are to elucidate how the immune system responds to aberrantly-glycosylated tumor associated carbohydrate antigens and how this is related to populations of $CD301b^+$ immune cells observed within the tumor microenvironment. Our first aim is to characterize a new population of $CD301b^+$ immune cells that accumulate within the tumor microenvironment. Next, we will determine the role of tumor glycans in accumulation of the $CD301b^+$ cell population. Finally, we aim to overcome breast cancer progression through inhibition of TACA-$CD301b^+$ interactions.

We have utilized a murine breast cancer model to determine how populations of CD301b$^+$ immune cells change as tumors develop at the mammary site. Using this model, we have characterized CD301b$^+$ immune cell function and carbohydrate involvement. We have used flow cytometry to confirm key immune cell markers and cytokines produced by these CD301b$^+$ immune cells, as well as how glycan changes on the tumor cell surface correlate with CD301b$^+$ immune cell accumulation and suppressive function. This research will have significant impact on improving immunotherapy in breast cancer.

(133) Identification of glycosyl linkages in switchgrass biomass recalcitrant to *C. thermocellum* deconstruction

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Plant cell walls are a removable source of carbohydrates for the production of biofuels and biomaterials. The successful of this emerging bioeconomy requires complete solubilization and utilization of virtually all carbohydrate components in the plant biomass. Plant mechanisms have evolved to counteract the deconstruction of their cell walls by invading microbes. These include production of hydrolase-resistant glycosyl linkages or ultrastructural features. Identifying these structures and the catalytic activities required for their deconstruction will enable the production of biomass and microbes with improved characteristics.

In this study, we are identifying cell wall fragments that are solubilized but not fully hydrolyzed by *Colstridium thermocellum* growing on switchgrass biomass as carbon source. *C. thermocellum* is a thermophilic, saccharolytic microbe, which has an outstanding ability to achieve high solubilization of cellulosic biomass. We are using NMR methods in combination with other analytic techniques to identify and structurally characterize these recalcitrant oligomers.

The results of these analyses showed that glucuronoarabinoxylan oligosaccharides are the main cell wall fragments that accumulated in the broth during *C. thermocellum* fermentation. This polysaccharide accounts for 25% of total switchgrass dry biomass. The unbroken linkages in the recalcitrant oligomers were identified together with CAZyme activities needed for their hydrolysis. The application of this information to develop models to predict the behavior during microbial deconstruction of biomass from other species with similar cell wall structure will be discussed.

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(134) Siglec-8-Binding Keratan Sulfate Chains that Regulate Airway Inflammation

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Airway inflammatory diseases are characterized by infiltration of immune cells, which are tightly regulated to limit inflammatory damage. Most members of the Siglec family of sialoglycan binding proteins are expressed on the surfaces of immune cells and are immune inhibitory when they bind their sialoglycan ligands. When Siglec-8 on activated eosinophils and mast cells binds to its sialoglycan ligands, apoptosis or inhibition of mediator release is induced. We found that human airway ligands for Siglec-8 are sialylated and 6'-sulfated keratan sulfate (KS) chains on large proteoglycans. Purified Siglec-8-binding proteoglycans from human airways increase eosinophil apoptosis in vitro. Given the structural complexity of intact proteoglycans, target KS chains were isolated from postmortem human tracheobronchial tissue and from human nasal lavage. Biological samples were extensively proteolyzed, the remaining sulfated glycan chains captured and resolved by anion exchange chromatography, methanol precipitated then chondroitin and heparan sulfates enzymatically hydrolyzed. The resulting preparation consisted of KS chains attached to a single amino acid or a short peptide. Purified KS chains from tracheobronchial extracts and nasal lavage were hydrolyzed with either hydrochloric acid or trifluoroacetic acid to release acidic and neutral sugars, respectively, followed by carbohydrate analysis on a DIONEX ICS 6000. To isolate Siglec-8-binding KS chains, purified KS chains from biological samples were biotinylated at the amino acid, resolved by affinity and/or size-exclusion chromatography, the resulting fractions immobilized on streptavidin microwell plates, and probed for binding of Siglec-8-Fc. Siglec-8 affinity chromatography of the tagged KS chains was performed on a nickel column derivatized with a His-tagged pentameric form of Siglec-8. Most of the KS chains flowed through the column, whereas Siglec-8 binding was retained through 150 mM NaCl and eluted with 1.5 M NaCl. Based on size separation, Siglec-8-binding KS chains are high molecular weight (>40 kD) and do not co-elute with highly sulfated KS chains detected by mAb

5D4. Similarly, purified KS chains were cleaved with keratanase I, hydrazine-biotin tagged on their reducing ends, size-separated, and resulting fractions immobilized on streptavidin microwell plates. Keratanase I-cleaved KS chains showed a Siglec-8-binding peak at ~2 kD, indicating that the longer Siglec-8-binding KS chains contained keratanase-I-sensitive mono-sulfated LacNAc repeat(s) with a terminal oligosaccharide epitope resistant to keratanase I which retained Siglec-8 binding. These data reveal a minor fraction of terminally sialylated KS chains as the Siglec-8 ligands in human airway tissues and secretions. Characterizing the human airway Siglec-8 ligand may provide drug leads for treating airway inflammatory diseases such as asthma and COPD. Supported by NIH grants U01AI136443, P01HL107151, and T32GM080189 (to RNP and AGG).

(135) Extracellular ST6Gal-1 inhibits MCSF-mediated monocyte maturation into macrophages

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Resident macrophages are diverse populations of specialized immune cells present in almost every organ of the body, and are essential for the onset of inflammation in response to pathogens and maintaining immune homeostasis at steady state. The macrophage colony stimulating factor (MCSF) is the main cytokine responsible for maintaining resident macrophage populations, and plays a role in the maturation of inflammatory monocytes into macrophages in response to inflammatory stimuli. Previous studies from this laboratory demonstrated the ability of extracellular ST6Gal-1 to attenuate GCSF (granulocyte colony stimulating factor)-dependent generation of granulocytes by impeding the transition from the common myeloid progenitor (CMP) into the granulocyte progenitor (GP) stage of granulocyte production. Here we present data supporting an additional checkpoint for extracellular ST6Gal-1 in myelopoiesis, that is in the MCSF-dependent transition of monocytes into mature F4/$80^{hi}Ly6C^{low}$ macrophages. Internalization of the MCSF receptor in response to MCSF was also inhibited in the presence of ST6Gal-1. The resulting cells exhibit monocytic rather than macrophage-like characteristics, and decreased ability to secrete cytokines upon challenge with lipopolysaccharide(LPS). Subsequent polarization of these cells into prototype inflammatory M1 macrophage or anti-inflammatory M2 macrophage is also affected, with ST6Gal-1 treated cells displaying a transcriptional profile distinct from control cells. Furthermore, we have observed increased numbers of resident macrophage populations in the peritoneal cavity of global ST6Gal-1 knock out mice, supporting a role for ST6Gal-1 in monocyte-macrophage functionality and homeostasis *in vivo*, with implications in controlling inflammatory responses *in vivo*. (This work was supported by the Program of Excellence in Glycosciences P01-HL107146).

(136) Using HSC70 as a proxy to identify c-mannosylated proteins in RAW264.7 cells exposed to lipopolysaccharides

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In eukaryotes, C-linked glycosylation of tryptophan can occur at the C_2 atom of the indole side chain via a carbon-carbon glycosidic bond with α-mannose. Presently, it is known to occur on proteins that contain the Trp-Xaa-Xaa-Trp/Cys motif (Xaa = any amino acid) found in the Thrombospondin Type-1 Repeat (TSR) domain as well as in Type I cytokine receptors. C-mannosylation has been implicated to be involved in cellular secretion and, intriguingly, the macrophage lipopolysaccharide (LPS) signaling pathway. This well-understood pathway triggers a cascade resulting in the release of tumor necrosis factor α (TNFα) upon recognition of LPS by Toll-Like Receptor 4 (TLR4) at the macrophage cell surface. Prior work has shown that C-mannosyltrytophan-containing peptides enhance the TNFα output from RAW264.7 macrophage-like mouse cells that are co-stimulated with LPS. In addition, interaction of Hsc70 with the C-mannosylated peptide is necessary for this effect. Our goal has been to identify the protein ligand(s) of Hsc70 responsible for this regulatory effect. RAW264.7 cells were cultured and treated in the absence or presence of 1 μg/mL LPS for 0 – 24 hours. Secreted proteins were collected by precipitating the conditioned medium and membrane and cytosolic proteins were collected by lysing scraped cells and precipitating the solubilized proteins. After performing immunoprecipitation using a commercially available anti-mouse Hsc70 primary antibody, eluted fractions were separated on SDS-PAGE and stained in silver nitrate. Approximately 115 visible bands were excised, proteolytically digested with trypsin and chymotrypsin and sequence analyzed using nanoLC-MS/MS on a Q Exactive Plus triple-quadrupole/orbitrap mass spectrometer. Using this method, 1174 unique protein hits were identified as possible Hsc70 ligands; 295 of which were only detected in LPS-stimulated cells. 39 proteins from this cohort have Trp-Xaa-Xaa-Trp/Cys consensus sequons which are currently being verified through mass spectrometry. In addition, we have observed peptides which suggest C-mannosylation in proteins in trytophans in non-consensus sequons.

(137) HIV-1 Envelope N-glycan Shield

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HIV-1 envelope (Env) is the sole surface protein of HIV-1 and initiates entry of virus into host cells. Approximately 90

N-glycans form a glycan shield that is the primary interface between the virus and host immune system, yet how individual N-glycosylation site (NGS) mutations coordinate to evade the immune system through mutation is not well understood. Using high-resolution mass spectrometry, we can track shifts in N-glycan heterogeneity due to mutations observed in immune escape viral variants. The shifts in heterogeneity defined a range of NGS sequons confined to a structural region that we used for a mutation cluster analysis using the HIV-1 LANL Database. This resulted in a finite number of sequons within a microdomian and created an immune escape map of conserved and variable glycans. A 500-ns molecular dynamics simulation of the same viral variants provided further insight in the structural dynamics of the glycan movements within each microdomain that serve to shield Env. We tested how shifting the number of sequons within one microdomain (high-mannose patch) could affect Env functionality. Four Env variants that had either 5, 4, 4, or 3 sequons were analyzed using an infectivity assay, a CD4 binding assay, and antibody neutralization assays. These studies provide detail on how we can identify Env N-glycan microdomains and utilize these microdomains to predict viral escape mutations.

(138) Characterizing the terminal GalNAc O-glycoforms recognized by lectins in the context of Clustered IgA1 O-glycosylation

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Human immunoglobulin A1 (IgA1) contains both *N*- and *O*-glycans. The *O*-glycans of IgA1 are clustered together in the Ser-, Thr-, and Pro-rich hinge region between the first and second constant domains of the heavy chain. IgA1 is found in the circulation as well as in the mucosal tissues and secretions and exists in multiple molecular forms. The different molecular forms of IgA1 may exhibit differential *O*-glycosylation. Native IgA1 usually has a range of three to six *O*-glycans. The *O*-glycans of the IgA1 hinge region usually consist of serine (Ser) and threonine (Thr) with *N*-acetylgalactosamine (GalNAc) attached to a galactose (Gal) by a β1,3-linked glycosidic bond. Both the GalNAc and Gal may also be sialylated. However, sera IgA1 from with patients with IgA nephropathy (IgAN) is characterized by the presence of Gal-deficient *O*-glycans in the hinge region that can act as epitopes for anti-glycan IgG antibodies. These glycoforms, present in elevated amounts in the circulation of IgAN patients, are recognized by Gd-IgA1-specific IgG autoantibodies, resulting in the formation of pathogenic immune complexes. Our group has established a quantitative ELISA test for detection of Gd-IgA1 using GalNAc-specific lectins from *Helix aspersa* (HAA) or *Helix pomatia* (HPA). Using this assay, we and others have shown that most patients with IgAN have elevated amounts of circulatory Gd-IgA1. However, it remains unclear which *O*-glycoforms of IgA1 are recognized by the lectins. Both HAA andHPA recognize terminal GalNAc, but not sialylated or galactosylated GalNAc. The assay is further complicated by the fact that IgA1 exists in multiple forms in serum. Monomeric IgA1 is produced by bone marrow and comprises approximately 95% of total serum IgA1. Polymeric forms of IgA1 are produced by mucosal tissues and represent < 4-5% of total IgA1 but has the potential to have more sites of terminal GalNAc per molecule. The remaining balance may be IgA1 bound in immune complexes. In this study, we will describe the results of analyses of different molecular forms of serum IgA1 by the lectin ELISA test as well as by high-resolution mass spectrometry profiling. We are using IgA1 isolated from sera of IgAN patients and healthy controls to gain insight into the nature of the lectin binding terminal GalNAc in the context of clustered sites of *O*-glycosylation. Our current results suggest that polymeric IgA1 is recognized by GalNAc-specific lectins more readily than monomeric forms. These results will be compared with high-resolution mass spectrometry profiles of IgA1 hinge-region glycopeptides. Moreover, we will determine whether masking of terminal GalNAc by sialic acid may provide an indirect assay for the extent of GalNAc sialylation. These results and others from the extensive MS profiling of the different molecular forms of IgA1 will be presented.

(139) Integrin αM and the Macrophage Mannose Receptor in secreted protein turnover and disease

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N-glycosylation is a major post-translational modification of secreted and cell surface proteins. We have shown that N-glycan structures are markers of protein aging and participate in determining glycoprotein half-lives in blood circulation. The combined roles of glycosidases and endocytic lectins receptors establish this intrinsic mechanism, which can be manipulated, and they further participate in disease pathogenesis including inflammation and coagulopathy during sepsis. Circulating basal levels of exo-glycosidases including neuraminidases, beta-galactosidases and glucosaminidases have hydrolytic activities that are normally involved in an intrinsic stepwise unmasking of underlying and otherwise cryptic glycan structures sequentially at the termini of N-glycan branches as the nascent protein ages. These aged N-glycans include ligands of various endocytic lectins, including the Ashwell–Morell receptor (AMR), integrin αM (ItGam), and macrophage mannose receptor (Mmr). An accumulation of identified plasma glycoproteins bearing ligands for galactose-binding ECA and RCA lectins was previously observed in AMR deficiency.

Mammalian endocytic lectin receptors with agalacto- and mannose-binding activities have also been identified, including ItGam and Mmr, respectively. Mice lacking ItGam and Mmr appear to accumulate glycoproteins in the blood that bear N-acetylglucosamine (GlcNAc) and mannose (Man) ligands, respectively. We are investigating the repertoire of proteins altered by increased life-span and abundance in the absence of these two lectin receptors. We are further comparing these findings with those using pharmacological inhibitors of galactosidase and glucosaminidase activities. These lectin receptor deficiencies along with the corresponding glycosidase inhibitors are also being investigated in the context of inflammation and coagulopathy in the pathogenesis of sepsis, by the identification of altered proteomes that are linked to glycoprotein half-lives, abundance, and function determined by the ItGam and the Mmr lectins.

(140) Analysis of Released N-Glycans and Glycopeptide Profiling of Prostate Cancer Tissue

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Introduction: Glycosylation is a complex posttranslational modification that plays a role in many biological processes. Glycosylation expression is aberrant in many types of cancer, making it an attractive source for potential biomarkers and therapeutics targets. Increases in sialylated, fucosylated, and highly-branched glycans have been observed in prostate cancer tissue and patient plasma, however characterization of the microheterogeneity at specific glycosites of prostate tissue glycoproteins is lacking. Describing glycosylation is key to understanding its role in cellular functions that promote tumorigenesis. In this study, mass spectrometry-based intact glycopeptide analysis along with released N-glycans analysis are used to characterize glycosylation patterns in prostate cancer and normal tissue.
Methods: Fifteen pairs of prostate cancer tissue and patient-matched normal tissue were obtained from men who underwent radical prostatectomies. Proteins were extracted from the lysed cells of homogenized tissue. For glycopeptide analysis, proteins were denatured, reduced, alkylated, and digested with trypsin. The resulting digest was enriched for glycopeptides using amide-HILIC solid phase extraction. Glycopeptides were then analyzed by nano LC-MS/MS. MS1 scans were performed over m/z400-1800, and the top five most intense ions (2+ charge state) were subjected to HCD fragmentation. For global N-glycan profiling, glycopeptides prepared as described above were rapidly deglycosylated and labeled with fluorescent dye InstantPC (ProZyme), and analyzed by LC-MS.
Preliminary Data: Proteins were extracted from fifteen cancer/normal pairs of prostate tissue, digested, and enriched for N-linked glycopeptides using HILIC SPE cartridges packed with hydroxyethyl amide silica. During LC-MS analysis, glycopeptides are subjected to suppression effects of the far more abundant non-glycosylated peptides, especially in complex protein mixtures from clinical samples, making a glycopeptide enrichment step necessary to achieve adequate signal. An average of 125 glycopeptides were identified per patient from 70 unique glycoproteins, although the number of glycopeptides identified per sample varied. Glycopeptide identifications provide protein identification, glycosylation site localization, and glycan composition. Glycomic profiling was performed on both pooled normal and pooled prostate cancer samples to corroborate the glycan compositions identified in the glycopeptide analysis. Preliminary results reveal the identification of a diverse set of unique glycan structures in normal prostate tissue. Biantennary structures HexNAc4Hex5Neu2 and high-mannose structure HexNAc2Hex5 (Man5) were the most abundant species and were also frequently identified in the intact glycopeptide analysis. Tri-sialylated species (HexNAc5Hex6Neu3 and HexNAc5Hex6Neu3Fuc1), and a series of high mannose structures from Man3 to Man7 were also identified in both glycomic and glycopeptide analysis.

(141) A single Fc g receptor IIIb / CD16b amino acid distorts the structure upon binding immunoglobulin G1and reduces affinity relative to CD16a

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Fc γ receptors (FcγRs) expressed on the surface of innate immune cells bind immunoglobulin G (IgG) through the crystallizable fragment (Fc). Recognition of Fc coating a pathogen targets its destruction by the innate effector cell. FcγRs are grouped into high affinity (FcγRI / CD64)and low affinity (FcγRII / CD32 and FcγRIII/ CD16) varieties. FcγRs and IgG are heavily modified with N-glycans that have been shown toimpact function and structure. CD16 has two main isoforms expressed on either neutrophils, the lower affinity CD16b,or monocytes and NK cells, the higher affinity CD16a. The factors contributing to this affinity difference are unknown because the two receptors are nearly identical, differing by 4 amino acid residues. Here we define a single amino acid residue at position 129 (G or D) that accounts for the difference between CD16a and CD16b affinity. Furthermore, we solved the first structure of N-glycosylated CD16b in complex with afucosylated IgG1-Fc that shows D129 deforms the receptor backbone upon binding disrupting the binding surface between the C'E loop of IgG1 Fc. G129 of CD16a in complex with IgG1-Fc, however, is similar in structure to the free, nonbinding form of CD16 suggesting the D129 residue forces CD16b to adopt a strained conformation upon binding.Overall these data provide

valuable information for the design of new CD16b-specific targeting therapeutics.

(142) Development of new soluble Siglec constructs to better understand regulation of immune cells by Siglecs

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The ability of immune cells to distinguish between '*self*' and '*non-self*' is critical to human health. Immune cells use a wide range of activatory and inhibitory receptors to guide self/non-self discrimination by recognizing molecular patterns that are unique to non-self and self, respectively. Siglec (sialic acid-binding immunoglobulin-type lectins) are a family of immune cell receptors whose ability to modulate immune cell function is intimately tied to interactions with sialic acid-containing glycoconjugates. Although much has been learned about the specific glycans that serve as Siglec ligands, there are still many unknowns. To better understand the nature of the glycan ligands of Siglecs, new tools and approaches are required. A common approach to studying Siglec-ligand interactions in solution is to use Fc-chimeric versions of Siglecs consisting of the first 2 or 3 domains of the Siglec fused to the Fc region of an antibody. While this platform has advanced our knowledge of glycan ligands of Siglecs, standard constructs have limitations. Here, we present a new versatile set of Siglec-Fc chimeras. Specific features of these new constructs include affinity tags for purification and detection, mutations in the Fc domain that render the Fc region incapable of binding Fc-receptors on immune cells, a proteolytic site between the Siglec and Fc regions to enable the production of monomeric Siglecs, and a consensus sequence for enabling site-specific labeling. Progress will be presented for ongoing applications using these new constructs in innovative ways to ultimately provide a better understanding of the glycan ligands of Siglecs.

(143) Using a CRISPR-Cas Whole-Genome Screen to Identify Genetic Factors Linked to Expression of Cancer-Associated O-glycans

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Truncated O-glycans, designated Tn and STn, have been recognized as cancer-associated antigens correlated with worsened patient outcomes for more than four decades. In epithelial cancers, truncated O-glycans are identified in 70-90 % of cases and are often seen in premalignant lesions, making them attractive therapeutic targets as well as interesting biological entities potentially linked to fundamentally important processes in carcinogenesis. However, little is still known of the exact mechanism(s) resulting in the expression of truncated O-glycans in cancer cells. Several hypotheses have been proposed, including mutations of the *CORE1* and *COSMC* genes, pH-dependent regulation, methylation of gene promotors, relocation of GalNAc-transferases in the secretory pathway and substrate deficiencies, but none of these hypotheses have yet to explain more than a limited subset of cases. To investigate potential genetic factors linked to Tn and STn expression, we therefore conducted a whole-genome genetic perturbation screen in the human keratinocyte HaCaT cell line with stable expression of the Cas9-protein and no expression of truncated O-glycans. Employing the glycan-specific monoclonal antibodies to Tn (mAb 5F4) and STn (mAb 3F1), we subsequently used FACS to separate cells with perturbated genes resulting in Tn or STn expression from keratinocytes not expressing truncated O-glycans. We found a subset (approximately 0.05 %) of the genetically modified cells from the whole-genome screen to be Tn- or STn-positive when analyzed på flow cytometry, with a larger proportion of Tn-positive cells as compared to STn-positive cells. We are currently using Next Generation Sequencing (NGS) on PCR products amplified from the signature sgRNA sequences imbedded in the lentiviral inserts from the Brunello library to identify candidate genes which – when knocked out – results in expression of truncated O-glycans in the Tn- and STn-positive cell populations. We aim to validate the candidate genes by conducting single-gene knock outs of the genes targeted by the most abundant sgRNAs in the Tn- and STn-positive cell populations. Subsequently, we seek to evaluate the single-cell knock outs by antibody staining, lectin staining and MS/MS to confirm the expression of Tn- and STn-antigens, as implied by the findings in the whole-genome screen. In conclusion, we conducted a whole-genome screen investigating the effect on expression of truncated O-glycans from perturbation of single genes by CRISPR-Cas9 induced knock outs in a human cell line. We find that the whole-genome screen results in expression of Tn- and STn-expression in a small number of cells – but a larger number than what can be explained from *COSMC* and *CORE1* perturbations exclusively – and aim to present candidate genes and validated genetic targets involved in expression of truncated O-glycans in a human setting.

(144) CGE-LIF as a novel approach for the analysis of glycosphingolipid-derived glycans

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Analysis of glycosphingolipid (GSL) glycosylation remains a major challenge in analytics. Current technologies, such as mass spectrometry (MS) or reversed-phase (RP)-HPLC are time-consuming and mostly low-throughput. Here we present a novel high-throughput-compatible approach for the analysis of GSL-derived glycans using capillary gel electrophoresis coupled to laser-induced fluorescence detection (CGE-LIF), which has so far been widely applied by us and others for N-glycan profiling. We show for the first time the adaption of CGE-LIF for the analysis of GSL glycans. GSL glycan head groups were efficiently released from the ceramide by digestion with ceramide glycanase, fluorescently labelled and could subsequently be separated by CGE-LIF. Using defined glycans, we built up a data base that currently comprises the migration times of 36 different glycan structures belonging to ganglioseries, globo- / isoglobo series and lacto- / neolacto-series. The separation power of CGE-LIF is capable to not only discriminate glycans differing in the number or type of monosaccharides but also clearly distinguishes peaks of structural isomers that differ either in the positioning of identical monosaccharides (e.g. GD1a-derived glycan vs. GD1b-derived glycan) or even in the type of linkage between identical monosaccharides (e.g. fucosyl lactotetra vs. fucosyl neolactotetra).

We applied this novel approach for the analysis of GSL glycans of human induced pluripotent stem cells (hiPSCs). Based on our database, 14 different glycans could clearly be assigned and were confirmed by exoglycosidase digests. The majority of peaks could still be detected from as few as 10^5 cells. Subsequently we quantitatively compared GSLs of hiPSCs and cardiomyocytes (CMs) derived thereofby *in vitro* differentiation. This analysis revealed that levels of GSL-derived glycans with globo- (sialyl globopentaose / stage-specific embryonic antigen 4 (SSEA4), globopenta / SSEA3, globotetra) and lacto-series (fucosyl lactotetra / SSEA5, lactotetra) core structures were considerably reduced, whereas levels of ganglioseries glycans (GD3-derived glycan and GM3-derived glycan) were strongly augmented upon differentiation of hiPSCs.

Taken together, once having established a comprehensive migration time database, CGE-LIF provides a cheap, easy-to-use and fast access for the analysis of GSL glycans with superior sensitivity and specificity that can be simply scaled up to a high-throughput approach.

(145) Inhibition of mucin type O-glycosylation by N-thioglycolyl-D-galactosamine affects lateral mobility of CD43 and formation of immune synapse

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Mucin-type *O*-glycans (MTOG) is abundantly found on many CD antigens on the surface of immune cells. MTOG mediate cell-cell, cell-pathogen, cell-antibody, and cell-extracellular matrix communications. Apart from imparting a large hydrodynamic volume and resistance to hydrolysis by proteases, MTOG play important roles in regulating lateral interactions between CD antigens. CD43 (leukosialin/sialophorin), in particular, is known to carry 80-90 MTOG on its extracellular domain which imparts rigid rod-like structure that projects up to 50 nm on the cell surface. CD43 is considered as a negative regulator of immune activation as it is able to sterically prevent establishment of cell-cell contacts. Previous studies have shown that CD43 is readily excluded from the immune synapse contact surface between a T-cell and an antigen-presenting cell (APC). Biophysical studies have established that the long surface molecules (> 50 nm in length) must be segregated out so that interaction of short surface molecules (10-20 nm projections in length) such as peptide-loaded MHC and T-cell receptors (TCR) could be facilitated leading to immune activation.

Earlier we have shown that peracetyl *N*-thioglycolyl-D-galactosamine (Ac_5GalNTGc (**1**)) inhibited MTOG (Agarwal, K., *et al.*, *J. Am. Chem. Soc.*, **135**, 14189-97 (2013)). Particularly, treatment with **1** resulted in hyposialylation and hypoglycosylation of CD43 while the polypeptide levels remained unchanged. We hypothesized that the readiness of CD43 to be sorted on the cell surface must be due to the abundance of MTOG. MTOG across the backbone prevents the relatively strong lateral protein-protein interactions thus enabling flexible movement on the plasma membrane. Consequently, inhibition of MTOG on CD43 by **1** may result in altered lateral mobility thus affecting the establishment of immune synapse. We will present results of our investigation on modulation of MTOG by **1** and its consequences on immune functions. We adapted an established model for formation of immune synapse using C57BL/6-Tg (TcraTcrb)1100Mjb/J (OT-1) transgenic mice which express the cognate TCR against the ovalbumin peptide SIINFEKL on CD8+ cells. T-cells were sorted and incubated under multiple conditions. Macrophages were pulsed with SIINFEKL and treated with metabolically engineering T-cells to allow formation of immune synapse. Confocal immunofluorescence microscopy was performed after staining of the immune cell complexes for CD43 (clone S7) and intercellular contacts, conjugates, and CD43-excluded conjugates were enumerated. Additional markers of T-cell activation such as CD25 and CD69 were investigated using flow cytometry. Our results show that inhibition of MTOG induced by **1** significantly reduces the formation of immune synapse compared to controls. Our small-molecule based approach provides a potential avenue to modify autoimmune disease conditions wherein the immune system is in a perpetually activated state.

(146) Differential Sialic Acid Binding Patterns of Phylogenetically-related Bacterial Exotoxin B Subunits Elucidated by Sialoglycan Microarray Analysis

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Glycan microarrays are critical tools for high-throughput analysis of glycan-protein interactions. We use this powerful technique with a focus on naturally occurring non-reducing terminal sialyltrisaccharides. Chemoenzymatic synthesis generated a diverse library of ~200 structurally defined sialyltrisaccharides, terminating in the common vertebrate sialic acids(Sias) Neu5Ac, Neu5Gc and Kdn, and some of their modified forms. Considering the size of our glycan library, microarray data sorting and analysis poses a major hurdle for high-throughput studies without a proper numbering system. We devised a numerical bar-coding system that assigns a unique code for individual glycans. The terminal Sia and its underlying monosaccharides are assigned from the non-reducing to the reducing end, each with three digits to describe a monosaccharide, its modifications, and its linkage. This simple bar code helps to sort glycans in logical ways, to optimize the printing process, and in motif searching.

The bacterial AB_5family of toxins are comprised of a cytotoxic A-subunit and pentameric B-subunit that bind terminal glycans on host cell surfaces. These toxins are major virulence factors for several pathogens. Phylogenetic analysis of known B-subunits that bind terminal Sias along with related molecules in available genomes showed a poor correlation of binding with bacterial species phylogeny. For example, the B-subunit of *Yersinia pestis* (YpeB) which shares a high degree of identity/similarity (58%/79%) with that of the *E. coli* subtilase cytotoxin (SubB), has very different Sia-binding patterns: while SubB preferably binds Neu5Gc-terminal glycans, YpeB has a much more diverse (Neu5Ac and Neu5Gc) binding domain. Further studies revealed that the related B-subunit from *Yersinia enterocolitica* (YenB) not only binds to all Neu5Ac and Neu5Gc glycans but also shows higher binding in similar concentrations. Differential Sia-binding patterns were observed when other phylogenetically related B-subunit from *S. Typhi* (PltB), *S. Typhimurium* (ArtB),*Vibrio cholerae* (CtxB), the cholera family homologue of *E. coli* (EcxB), and *E. coli* heat-labile enterotoxin (EtxB) were tested and the data sorted using our barcoding system.

In setting up this system, we noted that the theoretical population of sialoglycan trisaccharide sequences is > 205 million. Filtering out impossible combinations and focusing on known and likely sequences, the number tentatively dropped to ~10^6 possible sialyltrisaccharides in nature. While we developed this system for linear trisaccharides, it is notable that amplifying the calculation to a biantennary *N*-glycan with two terminal sialoglycan trisaccharide sequences results in squaring of possibilities, to >10^{12} potential combinations.We suggest that while glycomic approaches are addressing the challenges arising, naturally occurring probes like these B_5 toxins can be used by others to detect and track the sialome in biological systems of interest.

(147) Galectin-3 and N-acetylglucosamine promote myogenesis and mitigate the burden of Duchenne muscular dystrophy

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The muscle membrane, sarcolemma, must be firmly attached to the basal lamina. The failure of proper attachment results in muscle injury, which is the underlying cause of Duchenne muscular dystrophy (DMD), where mutations in the dystrophin gene disrupt the firm adhesion. In DMD patients, even moderate contraction causes damage, leading to progressive muscle degeneration. The damaged muscles are repaired through myogenesis. Consequently, myogenesis is highly active in DMD patients, and the repeated activation of myogenesis leads to the exhaustion of the myogenic stem cells. Therefore, approaches to reducing the risk of the exhaustion are to develop a treatment that strengthens the interaction between the sarcolemma and the basal lamina, and increases the efficiency of myogenesis. Galectin-3 is a solubleß-galactoside-binding protein and known to be involved in cell-cell interactions and cell-matrix interactions. Galectin-3 is expressed in myoblasts and skeletal muscle while its function in muscle remains elusive. We recently found evidence that galectin-3 and*N*-acetylglucosamine (GlcNAc), which increases the synthesis of binding partners (oligosaccharides) of galectin-3, promotes myogenesis*in vitro*. Moreover, in a mouse model of DMD,*mdx*mice, 10 day-treatment with GlcNAc significantly increased the muscle force production.The results suggest that treatment with GlcNAc may be a simple and effective treatment for DMD.

(148) Keratan sulfate chains released from human airways support Siglec-9 binding – comparison with Siglec-8 binding

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Siglecs are sialoglycan binding proteins most of which are expressed on the surfaces of immune cells, and most of which are immune inhibitory when they bind to complementary sialoglycan ligands. Relevant to human airway inflammation, Siglec-9 is expressed primarily on neutrophils and monocytes and Siglec-8 on eosinophils and mast cells where they drive different types of pathogenic lung inflammation. Evidence suggests that distinct endogenous sialoglycans on human airways and airway secretions engage Siglec-9 and Siglec-8 to dampen and thereby limit inflammatory damage. In extracts of postmortem human airway tissues (trachea/bronchus) and in human airway secretions (nasal lavage) we identified Siglec-9- and Siglec-8-binding sialoglycans that we resolved and characterized using a combination of size exclusion chromatography, composite agarose-acrylamide gel electrophoresis, blotting, and probing with Siglec-9-Fc or Siglec-8-Fc chimeras. Siglec-9-Fc and Siglec-8-Fc binding to distinct sets of high molecular weight proteins was reversed by sialidase and also by keratanases I and/or II, indicating that human Siglec-9 and Siglec-8 ligands from human airways require sialylated keratan sulfate (KS) chains. In the current study, KS chains were released from postmortem human tracheobronchial tissue and from human nasal lavage and probed for Siglec-9 and Siglec-8 binding. Biological samples were extensively proteolyzed, the remaining sulfated glycan chains captured and resolved by anion exchange chromatography, methanol precipitated then chondroitin and heparan sulfates enzymatically hydrolyzed. The resulting preparation consisted of KS chains attached to a single amino acid or a short peptide. For Siglec binding analyses, KS peptides were tagged at the amino acid with biotin, resolved by affinity and/or size exclusion chromatography, the resulting fractions immobilized on streptavidin microwell plates and probed for binding of Siglec-9-Fc and/or Siglec-8-Fc. Siglec-9 affinity chromatography of the tagged KS chains was performed on a nickel column derivatized with a His-tagged pentameric form of Siglec-9. Most of the KS chains flowed through the column, whereas most Siglec-9 binding was retained and eluted broadly with stepwise 150 mM NaCl and 1.5 M NaCl. Fractions eluted from the Siglec-9 affinity column failed to bind to Siglec-8. When the same mixture of KS chains was resolved on a Siglec-8 affinity column, most of the KS chains flowed through the column whereas all of the Siglec-8 binding KS chains remained on the column until eluted with 1.5 M NaCl. The Siglec-8 bound fractions also bound Siglec-9 when immobilized on streptavidin wells, but were not retained when passed over a Siglec-9 affinity column. We conclude that Siglec-9 and Siglec-8 bind to distinct sets of sialylated KS chains from human airways. Supported by NIH grants U01AI136443, P01HL107151, and T32GM080189 (to RNP and AGG).

(149) Breaking the Allergic Cascade by Modulating IgE Glycosylation

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The prevalence of allergies has markedly increased over the past few decades, constituting a major healthcare problem. Allergies are caused by production of immunoglobulin ε (IgE) antibodies targeting environmental substances, such as pollens, pet dander, and foods. Individuals become sensitized when allergen-specific IgE binds to the high-affinity receptor (FcεRI) on tissue mast cells or blood basophils. Subsequent allergen exposure crosslinks cell-bound IgE, resulting in the release of inflammatory mediators including histamine, leukotrienes, and prostaglandins. Despite its significance in allergic diseases, IgE biology suffers from major knowledge gaps. While IgE is critical for pathogenesis of allergic diseases, some individuals have allergen-specific IgE but do not experience allergic symptoms. Further, some individuals outgrow their allergens, while retaining detecting levels of allergen-specific IgE. Thus, despite its discovery over 50 years ago, it is not clear what make IgE pathogenic. The importance of glycosylation for a number of antibody classes has been established. However, the contribution of glycosylation to IgE biology is less clear. IgE are the most heavily glycosylated monomeric antibodies with seven Asparagine (N)-linked glycosylation sites on its constant domains. One site carries exclusively oligomannose glycans (N394), one site is unoccupied (N383), while the remaining sites contain sialylated glycans. We set out to define the role of IgE glycosylation in anaphylaxis, and using functional models of allergies, we showed that glycans play divergent and important roles in regulating allergic response. It is appealing to speculate that glycans on IgE explain the presence of allergen-specific IgE and manifestation of allergic symptoms and may serve as a potential allergic disease biomarker and a novel therapeutic target.

(150) Micropermethylation and subsequent LC-MS based profiling of glycoproteins by Tool for Rapid Analysis of glycopeptide by Permethylation (TRAP) method

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The comprehensive analysis of large glycoprotein sample sets will not be possible until the currently available, complicated analysis process can be significantly simplified. We recently developed a promising, simple workflow integrating glycomics and glycoproteomics in a single experiment by directly permethylating the protease digest of glycoproteins. A

high-resolution tandem MS/MS technique and subsequent data processing were employed for the characterization of the permethylated glycopeptide. The TRAP approach is also useful for the analysis of glycoproteins from the non-mammalian origin which are resistant to both enzymatic and chemical method of release. Herewith, we report glycoprotein characterization using TRAP strategy by performing the permethylation in a microscale which is suitable for reliable permethylation of glycopeptides from the protease digest of large sets of glycoproteins at a low amount. We optimized the micropermethylation reaction and sample purification conditions and thus improved the sensitivity and throughput of permethylation reactions in microscale. Subsequently, we injected the glycopeptides permethylated by TRAP method into an LC-MS/MS system through auto sampling and thereby characterized the glycan sequence and branching at the N-glycosylation sites. Our results on the analysis of glycoproteins such as monoclonal antibodies (mAb) and bovine RNase B, indicated that TRAP followed by LC-MS/MS enables characterization of glycan isomers and the types of glycoforms at each N-glycosylation site. Moreover, we could deduce the glycosylation linkages and monosaccharide compositions by the GC-MS analysis of the permethylated glycopeptides generated via TRAP. We are in the process of developing a cost-effective kit and a data analysis platform for the rapid glycoprotein analysis through TRAP strategy and this would enable convenient, rapid, and reliable high-throughput screening of protein glycosylation.

(151) Exploring the Role of APP Protein O-Glycosylation in Alzheimer's Disease

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The amyloid-b precursor protein (APP) is a transmembrane protein that can undergo proteolytic cleavage by three proteases, a-, b- and g-secretases, to determine its fate in Alzheimer's disease (AD) pathogenesis. The protein undergoes proteolysis by b- and g-secretases to produce amyloid-b peptides (Abs), which is a hallmark of AD. However, only ten-percent of the protein will follow this amyloidogenic pathway, whereas the remaining ninety-percent will undergo proteolysis by a-secretase and g-secretases, resulting in the non-amyloidogenic pathway. Post-translational modifications (PTMs) such as glycosylation are known to regulate protein conformation and can play an important role in the onset of this disease. Recently, it was found that the four threonine residues Thr 633, Thr 651, Thr 652, and Thr 659, in the vicinity of the b-secretase cleavage site (Met 671~Asp 672) of APP, are modified by complex mucin-type *O*-glycans. Moreover, an increase of up to 2.5 times in tyrosine glycosylation, located within the Ab42 region of APP (Tyr 681), was found in AD patients in comparison to the non-AD patients. These findings suggest the possible role of *O*-glycosylation in APP proteolytic processing and modulation of its fate to either the non-amyloidogenic or amyloidogenic pathway associated with AD. Therefore, our goal was to synthesize both *O*-glycosylated and non-glycosylated APP peptide analogs to understand the role of *O*-glycosylation on APP's conformation, proteolysis and aggregation. Our preliminary results show that the non-glycosylated peptide analogs in water show characteristics of b-sheet conformation, however, the presence of a site-specific *O*-glycosylation seemed to favor either random coil or a-helical conformation. Furthermore, the level of b-secretase activity significantly increases for the glycosylated analogs containing the Swedish mutation, (Lys670Asn and Met671Leu) compared to their non-glycosylated counterparts. Likewise, the glycosylated analogs impacted the protein's aggregation kinetics by decreasing its lag phase more significantly than its non-glycosylated counterparts. In conclusion, our preliminary results suggest that APP's site-specific *O*-glycosylation can induce a conformational change in the protein and subsequently affect its proteolytic processing fate towards either amyloidogenic or non-amyloidogenic pathway. Our long-term goals are to assess the role of certain patterns of multiple glycosylation sites on Ser or Thr along with Tyr that are in close proximity to the b-secretase cleavage site and within the Ab42 region of APP, respectively, paving the way for novel AD-modifying therapies.

(152) A new way to target bacteria: Hetero-multivalent binding of glycolipids to Pseudomonas aeruginosa

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Inspired by the nature of *Pseudomonas aeruginosa* (PA) adhesion to the host cells via binding between various types of bacterial lectins and different host glycolipids, we have developed an innovative targeted drug delivery system to enhance drug retention at the infection site. This involves a hetero-multivalent binding strategy that uses different types of host glycolipid ligands on a liposome to simultaneously bind with different types of receptors on a bacterium. Liposomal drug carriers were chosen because liposomes provide critical two-dimensional glycolipid mobility to facilitate multivalent interactions. Multiple glycolipid ligands on a liposome can self-organize to enable concurrent binding with different receptors on a bacterium. Because of the reduced dimension of ligand diffusion, the reaction rates of the subsequent bindings are at least 10^4 times higher than the first binding event. This rate-enhancement mechanism, called Reduction of Dimensionality (RD), dramatically increases the efficiency of multivalent binding. A key concept of the RD mechanism is that, upon completion of the first

attachment, even low-affinity ligands can now contribute in the ensuing liposomal binding with the bacteria because the binding rates of low-affinity ligands are enhanced by the RD process. Traditionally, the targeted drug delivery schemes have decorated drug carriers with high-affinity ligands only. The RD mechanism suggests that we can simultaneously utilize both high- and low-affinity ligands to achieve higher binding avidity and specificity. To demonstrate this concept, we have exploited the ability of PA to bind with eukaryotic cells to design a targeted liposomal drug delivery system for PA. We have successfully found a new PA-specific ligand, lactosylceramide (LacCer), from eukaryotic cells, and improved the liposomal retention rate up to 400% by blending LacCer with the other known PA-specific ligand, Gb3, *in-vitro*. In addition, we have demonstrated that these glycolipid decorated liposomes could also penetrate through physiological barriers and reach the infection sites in a thigh infected mouse model. Our *in-vitro* and *in-vivo* studies indicated that this unique bio-mimetic approach not only improves the targeting efficiency towards PA, but also reduces the off-target binding.

(153) Co-evolution of a typhoid toxin homolog and nontyphoidal Salmonella

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Typhoid toxin is a distinct A_2B_5 bacterial toxin, which is unique to typhoidal Salmonellae, *Salmonella enterica* serovar Typhi (*S.* Typhi) and *S.* Paratyphi. Administration of purified typhoid toxin to experimental animals recapitulated many of the characteristic symptoms of typhoid fever. However, typhoid toxin homologs with a small amino acid sequence variation are found in a few nontyphoidal Salmonella serovars causing self-limiting gastroenteritis, such as *Salmonella* Javiana. Here we show a three amino acids sequence variation on 'javiana toxin's receptor-binding subunit PltB causes a switch of javiana toxin's glycan receptor binding preference from glycans abundantly expressed on cells of the systemic site (where *S.* Typhi infection occurs, and both α2-3 and α2-6 sialosides are present) to glycans abundantly expressed on cells of the gastrointestinal tract (where nontyphoidal Salmonella infection occurs, and α2-3 sialosides are predominant). Consistently, the administration of javiana toxin to the systemic site causes little to no typhoid fever symptoms, but javiana toxin binds and enters intestinal epithelial cells. We predict that javiana toxin plays a critical role in assisting *S.* Javiana to establish long-term infection in intestinal epithelial cells, although the detailed mechanism remains to be characterized. These findings highlight the remarkable co-evolution of bacterial toxins for their specific need.

(154) A new assay measures transport of UDP-galactose into the Golgi of cells from patients with mutation in SLC35A2, encoding the UDP-galactose transporter

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SLC35A2 is an X-linked gene encoding the only human UDP-galactose transporter (UGT). CHO-Lec8 cells carry a null mutation in this gene which abolishes all UDP-galactose transport and in consequence galactosylated glycans.

Multiple physicians provided fibroblasts from eight SLC35A2-CDG patients with different mutations to measure UDP-galactose uptake. We used 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4-MUβGlc*N*Ac) as a membrane-permeable, saturating acceptor to measure the rate limiting transport of UDP-[^{3}H]galactose into the intact Golgi vesicles of permeabilized cells. The product, 4-MUβGlc*N*Acβ-[^{3}H]Gal, was purified on C18 columns and counted. UDP-galactose uptake was undetectable in CHO-Lec8 cells and was impaired to various degrees in all patient cells. Some patient lines contain variable proportions of wild type and mutant allele that must be assessed to accurately determine the contribution of the mutant allele. As an alternative, we apply the same assay to each mutant allele expressed in CHO-Lec8 cells.

Using this simple assay we could measure the K_m of both human and CHO UGT at 3.1 μM. Similar analysis of patient cells is underway. These studies should allow us to determine which SLC35A2-CDG patients might benefit from galactose supplement therapy. By selecting the appropriate glycoside acceptors, we can determine the K_m of other Golgi nucleotide transporters deficient in other patients.

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(155) Defining the OGT Interactome and its Role in X-Linked Intellectual Disability

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X-Linked Intellectual Disability (XLID) affects approximately 1 in 500 males in the United States. We have identified several mutations in the O-GlcNAc Transferase gene (*OGT)* that are causal for XLID, but the mechanism underlying the phenotype is unknown. OGT is an essential nucleocytoplasmic glycosyltransferase that modifies nuclear and cytosolic proteins with a single β-N-Acetyl-Glucosamine (O-GlcNAc). OGT has thousands of substrates and O-GlcNAc serves a diverse set of functions, including nutrient sensing, transcriptional regulation, and modulating synaptic function. The O-GlcNAc modification is considered analogous to phosphorylation, but unlike the hundreds of encoded kinases that phosphorylate substrates with high specificity, OGT is the only enzyme responsible for the O-GlcNAc modification within the mammalian cell. Therefore, the mechanism of OGT substrate selectivity is a major area of

interest. It is thought that the N-terminal tetratricopeptide repeats (TPRs), rather than the C-terminal catalytic domain, of OGT are responsible for OGT substrate selection, in part by the recruitment of partner proteins that target OGT to specific substrates/cellular domains. All of the OGT XLID variants being studied here are localized to the TPRs, leading to our hypothesis on the mechanism by which *OGT* mutations lead to XLID: that *rather than interrupting the stability or catalytic activity of OGT, the OGT XLID variants exhibit lost or abnormal protein-protein interactions and that these anomalous protein interactions lead to the XLID phenotype*. This hypothesis is supported by data from our lab demonstrating that all of the OGT XLID variants are thermally stable, catalytically functional, and kinetically comparable to the wild-type (WT) OGT. To test our hypothesis, we will use unbiased proteomic approaches to define the WT and XLID OGT interactomes and identify aberrant interactors. We have utilized a rat brain bait/fish assay to initially identify several WT OGT TPR interactors of interest as well as biologically relevant differential interactors between the WT and XLID OGTs. We will additionally use a set of immunoprecipitation-based and proximity-ligation proteomics to fully identify and validate the OGT interactome and differential interactomes in Cas9 modified neural precursor cells and cortical neurons, for both the WT and XLID OGT. This approach will not only identify putative mechanistic proteins underlying the XLID phenotype, but also identify a neural WT OGT TPR interactome, which has yet to be defined and is an essential resource for the field.

(156) Deciphering the glyco(phospho)peptide code of symptomatic Alzheimer's Disease

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Alzheimer's disease (AD) is a progressive disorder that begins long before clinical symptoms appear. Prior research has found that the asymptomatic (no symptoms, ASYM) phase of AD is linked to abnormal brain accumulations of the protein fragment beta-amyloid and the protein tau. These studies show protein deposition in ASYM brains and mostly similar overall histopathology despite little to no evidence of cognitive impairment. This, and other studies, suggests that other molecular mechanisms are involved in initiating and propagating the disease process. Posttranslational modifications of proteins, including glycosylation, are capable of modulating the behavior of proteins without altering their expression. Thus, *we hypothesize that the glycomes, including glyco(phospho)proteins and their associated O-GlcNAc, O-GalNAc, and N-glycans are different in ASYM versus AD patient samples*. Here, we analyzed the protein glycosylation of the dorsolateral prefrontal cortex from ASYM and AD post-mortem brains by lectin blotting and mass spectrometry. Our preliminary data demonstrates that there are significant changes in glycosylation, including O-GlcNAc, N-, and O-glycans in ASYM versus AD samples. In order to generate these analyses, we have developed, *de novo*, a robust workflow to process such samples. We also include the use of novel synthetic glycans and glycopeptides with all types of glycosylation that also incorporate phosphorylation features to provide quantitative information and serve as standards for mass spectrometry methods. These findings will provide new information to help identify novel biomarkers for AD and distinguish the glycophosphoproteomes of AD from asymptomatic brains. These data will also generate a usable and accessible database for researchers interested in AD brain glycomics.

(157) NGLY1 regulates Aquaporins: Implications in NGLY1 deficiency disorder

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Patients with mutations in *NGLY1* cannot make tears (alacrima), have global developmental delay, movement disorder and liver dysfunction. N-glycanase 1 (NGLY1) de-glycosylates 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.

We found that NGLY1-deficient mouse embryonic fibroblasts (MEFs) and NGLY1-patient fibroblasts were resistant to hypotonic lysis and that NGLY1-deficient MEFs swell slower than WT MEFs. Since aquaporins (AQP) transport water, we hypothesized that AQP levels might be altered in NGLY1-deficient cells. Indeed, AQP1 mRNA and protein were reduced in NGLY1-deficient MEFs. shRNA knockdown of AQP1 in WT MEFs decreased hypotonic lysis, linking AQP1 to hypotonic lysis. NGLY1 shRNA, CRISPR-based deletion and overexpression studies confirmed that NGLY1 regulates AQP1 and hypotonic cell lysis. Surprisingly, complementing NGLY1-deficient cells with catalytically inactive mutant of NGLY1 (C309A) restored hypotonic lysis phenotype in mutated HAP1 cells and AQP1 protein in MEFs. These results suggest that AQP regulation does not require NGLY1's enzyme activity. Next, we hypothesized that NGLY1 regulation of AQPs is mediated by a transcription factor. Using a luciferase-based reporter construct containing the first 1000 or 2000 bp mouse AQP1 promoter region, we found that luciferase activity was reduced by 50% in NGLY1-deficient MEFs. This data supports our hypothesis and identifies the AQP1 promoter as a region required for binding NGLY1-dependent transcription factor. Current efforts involve identifying these transcription factor/s by combining bioinformatics and molecular biology techniques.

In conclusion, we have not only identified a novel function of NGLY1 i.e. regulating AQPs but also it's first enzymatic activity independent function. These findings may help shed light on the pathogenesis of the NGLY1 deficiency disorder.

This work is supported by the Bertrand Might Research Fund and NGLY1.org.

(158) Truncated O-glycans Promote the Epithelial-Mesenchymal Transition and Stemness Properties of Pancreatic Cancer

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More than 80% of human adenocarcinomas express Sialyl Tn (STn, Neu5Ac a2-6 GalNAca-O-Ser/Thr) antigen, and in most cases the presence of STn correlates with poor prognosis and reduced patient survival. Overexpression of STn antigen has been observed on many epithelial cancer cells, but the highest frequency was observed in pancreatic ductal adenocarcinoma (PDAC). We demonstrated that expression of truncated O-glycans (Tn and STn) in PDAC are due to hypermethylation of Core 1 synthase specific molecular chaperone*COSMC*, and induced aberrant expression of Tn/STn antigens on mucin and other glycoproteinsenhancethe malignant properties of PDAC cells with an unknown mechanism. To investigate the mechanism by which truncated O-glycans enhance tumorigenic properties of PDAC cells, we have genetically deleted*COSMC*in human PDAC cells (T3M4 and Capan 2) to express truncated O-glycans. These truncated O-glycan-expressing PDAC (SimpleCells, SC) cells exhibited increased cell migration and invasion compared to wildtype (WT) cells.Also, we found an increased expression of mesenchymal markers N-cadherin, Slug and matrix metalloproteinases (MMPs) in tumors with truncated O-glycan-expressing PDAC SC cells as compared to WT cells. Conversely,we also found reduced expression of epithelial markers such as E-cadherin and claudin in SC cells. We observed a significantly increased cancer stem cell (side) population in tumors that express truncated O-glycans. Furthermore, orthotopic implantation of T3M4 SC into pancreases of nude mice resulted in significantly larger tumors compared to T3M4 WT cells implanted tumors. These results suggest that aberrant expression of truncated O-glycans in PDAC cells enhances the tumor aggressiveness through modulation of the epithelial-to-mesenchymal transitionand cancer cells stemness properties.

Keywords: *COSMC,Core-1 synthase, Truncated O-glycans, EMT, Stem cells and PDAC*

(159) Truncated O-glycans Promote Epithelial-Mesenchymal Transition and Stemness Properties of Pancreatic Cancer

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Keywords: *COSMC,Core-1 synthase, Truncated O-glycans, EMT, Stem cells and PDAC*

(160) Investigating role of IgG glycosylation in Fc γ receptor binding

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IgG play pivotal role in linking adaptive immune response and innate immune response. Over the last twenty year, many antibody based drugs have been approved for indications ranging from cancer, autoimmunity, allergy, transplantation, and allergy. IgG consist of two regions, the antigen binding region (Fab), and the crystallizable fragment region (Fc). The Fab portion binds to the antigens with high affinity, while Fc region interacts with different Fc receptors expressed on leukocytes to trigger effector functions, which

include antibody-dependent cytotoxicity (ADCC), opsonization of antigen, and complement-dependent cytotoxicity.IgG antibodies contains a highly conserved N-linked glycosylation site at A297 position on antibody Fc portion, which profoundly influences its structure and function.The glycan core is composed of*N*-acetylglucosamine and mannose. Further the core structure is extended by addition offucose, bisecting*N*-acetylglucosamine, galactose, and sialic acid residues. Thisglycan composition is highly heterogeneous irrespective of IgG subclasses and this phenomenon is well established in healthy and different disease states. There has been significant progress in developing a biomarker based on glycan composition for different disease states. IgG Fc glycan composition plays a critical role in modulating ADCC, CDC by modulating binding to different Fc receptors. For example, removal of a core fucose sugar resulted in increased ADCC activity with greater binding affinity to FcγRIIIA and successfully therapeutic a fucosylated antibodies are developed. Sialylation of the IgG Fc glycan impairs binding to some FcγRs, and is responsible to that general anti-inflammatory activity of high dose intravenous immunoglobulin (IVIG). Here, we set out to comprehensively define the contribution of IgG Fc sialylation to FcγR. Indeed, understanding how sialylation, and other glycosylation patterns on IgG Fc modulate FcγR binding is important for predicting effector functions, and for rationale design of therapeutic antibodies.

(161) The functions of circulating neuraminidases Neu1 and Neu3 in blood protein homeostasis

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The aging of secreted proteins in blood circulation has been linked to a glycolytic mechanism that controls the formation of endocytic receptor ligands with increased age. The mechanism includes the gradual removal of monosaccharides at the termini of N-glycan branches. The hydrolytic activity of neuraminidases present in circulation cleaves terminal sialic acid linkages, exposing underlying asialo-glycan structures recognized by endocytic lectins including the hepatic Ashwell-Morell receptor. Neuraminidases are widely distributed in vertebrates and microorganisms. Four mammalian neuraminidases, encoded by *NEU1-4*, have been identified and characterized. Mice deficient in the lysosomal resident Neu1 show molecular features resembling sialidosis, a severe human lysosomal storage disorder, whilst Neu3 deficiency does not result in an obvious disease phenotype. The subcellular localization of Neu3 has been shown to be the plasma membrane and endosomal compartments. The membrane attachment mechanism is not yet clarified and Neu3 is usually denoted as membrane-associated. Recent discoveries in this lab have identified both Neu1 and Neu3 circulating in the plasma of humans and mice. The origin of these circulating neuraminidases is not yet known as many cell types express them including activated platelets, monocytes, vascular endothelium, and the erythrocyte surface. Their release into circulation could be due in part to lysosomal secretion, cell surface release mechanisms including proteolysis, or exosomal expression. To identify the functions of endogenous neuraminidases in the bloodstream, mice lacking single or both *Neu1* and *Neu3* alleles are being generated for study. Preliminary data from mice deficient in *Neu1* will be presented and indicates that Neu1-deficiency reduces the rate of glycoprotein aging by de-sialylation in circulation. Similar studies with Neu3 deficiency in the determination of lectin ligand levels will also be presented. The plasma proteome is also under investigation to identify glycoproteins modulated by Neu1 and Neu3 function with studies of glycan remodeling linked to circulating glycoprotein half-lives and their abundance among mutant and normal mouse populations. Identification of the origin(s) and process of neuraminidase release into circulation would also provide insight into mechanisms that regulate protein aging and turnover in health and disease.

(162) The role of multivalency and surface diffusion of glycolipid in lectin-glycolipid recognition

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The distinct characteristics, multivalency and the diffusion of glycolipids on a cellular surface, make the lectin-glycolipid recognition principle inherently different from the classic antibody-antigen binding. Lectin-glycolipid binding is often semi-specific; thus, a lectin can bind to different glycan structures with different affinities. Most lectins bind to glycolipids via multivalent interactions, in which multiple binding domains in a single lectin simultaneously interact with multiple glycan molecules. Moreover, the glycolipid ligands can freely diffuse and rotate on the 2D fluidic cellular membranes, and therefore, self-organizing to enable multivalent interactions with the target lectins. This prompts that why does Nature, after billions of years of evolution, choose these special characteristics as an action principle to manage the glycobiology processes? Prior studies often hypothesized that multivalency allows the accumulated strength of multiple affinities, leading to an enhanced lectin binding avidity. However, we recently demonstrated that these special characteristics can lead to the hetero-multivalent binding phenomenon (i.e. a lectin simultaneously binds to at least two types of glycolipid ligands), resulting in the alternation of lectin binding behaviors, including avidities, capacities, and kinetics. Our findings suggested that cells probably use these characteristics to control the lectin binding, in order to regulate downstream biochemical reactions. The concept of hetero-multivalent binding provides the molecular basis of a popular hypothesis in glycobiology: lectins are pattern

recognition receptors, which recognize glycan epitopes based on their number, density, and spatial distribution, in addition to their molecular structures. Here, we have used kinetic Monte Carol (kMC) simulation to explore the influence of these characteristics on lectin binding behaviors. We have surveyed a number of conditions and identified the critical variables influencing lectin-glycolipid recognitions.

(163) Identification of glycan-mediated interactions on cell surface by metabolic labeling using a photocrosslinking sugar analog

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Cell surface glycans have multiple functions in various cellular processes, like cell-cell interaction, signaling transduction, and host-pathogen recognition. However, the limited methods to identify such interactions are impeding our understanding of how glycans function in these processes. Therefore, it is important to develop tools to probe these interactions. N-acetylglucosamine (GlcNAc) is a common element in cell surface glycans. Previous study shows that GlcNDAz-1-phosphate, an analog of GlcNAc-1-phosphate functionalized by a diazirine group, can be metabolized by mammalian cells expressing mutated UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) to generate UDP-GlcNDAz. Here, we show that GlcNDAz can be incorporated into cell surface glycans and thus may be utilized as a probe to identify glycan-mediated interactions on the cell surface. Result of glycopeptide analysis using HPLC-MS/MS demonstrates the presence of GlcNDAz in N-linked glycans. In addition, galectin-1 and cholera toxin B subunit are found to be crosslinked to cell surface glycoproteins through GlcNDAz. The crosslinked glycoproteins can then be identified by proteomic analysis. These results support the possibility of using GlcNDAz to study glycan-mediated interactions on the cell surface.

(164) Cellular and biochemical analysis of the single, recurrent de novo mutation in COG4 causing Saul-Wilson syndrome

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Saul-Wilson syndrome (SWS) is a rare skeletal dysplasia with progeroid features and primordial dwarfism. Recently, we found 14 SWS patients with an identical, heterozygous *de novo* variant (p.Gly516Arg) in COG4. The phenotype of SWS patients is different from that of patients with biallelic variants that cause COG4-CDG.We analyzed the effects of this *de novo* mutation in SWS patient-derived fibroblasts, and in COG4-null cells complemented with the wild type and SWS mutant alleles.

COG4 is one of 8 subunits in the Conserved Oligomeric Golgi (COG) complex involved in protein retrograde and anterograde transport between the endoplasmic reticulum (ER) and Golgi and within Golgi. Brefeldin A (BFA) interrupts normal transport, merging the ER and Golgi, but the Golgi reforms when BFA is removed. Compared to wild type, BFA accelerated retrograde transport in SWS cells, but it slowed normal anterograde transport when BFA was washed out. A trans-Golgi network marker (TGN46) also disappeared slightly faster in SWS cells compared to controls. The accelerated retrograde and slowed anterograde vesicle transport disturbed the dynamic equilibrium that decreased Golgi volume by 2.8-fold compared to control cells.

Distribution of each COG subunit between cytoplasm and Golgi is unaltered in SWS cells, but COG4 protein increased 1.8-fold in SWS cells compared to controls, indicating the SWS variant is probably more stable. A portion of the cytosolic COG4 shifted to a higher molecular weight fraction in glycerol gradient centrifugation analysis in SWS cells, indicating that a portion of COG4 forms larger complexes compared to control cells.

Decorin, a proteoglycan present in the extracellular matrix of connective tissue and cartilage, was significantly affected in SWS individuals compared to controls. Both intra- and extracellular decorin from affected individuals showed a greater proportion of higher molecular weight forms compared to control cells, suggesting longer or more highly sulfated glycosaminoglycan (GAG) chains. A smaller proportion of intracellular decorin contained extended GAG chains in affected individuals when compared to controls.

To further investigate the variant's stability and function, wild type COG4 and the SWS mutant allele were transiently expressed in HEK293T COG4 knockout (KO) cells. The SWS COG4 allele was at least 2-fold more stable compared to wild type COG4. In addition, glycerol gradient centrifugation showed that a greater portion of the SWS allele is found in larger complexes in HEK293T COG4 KO cells expressing the SWS mutant alone or together with wild type COG4.

Our findings provide a mechanistic understanding of SWS at the molecular and cellular level, and help elucidate the pathogenesis of this rare skeletal dysplasia.

(165) Broad cytoprotective functions of plant-produced asialoerythropoeitin

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Enzymatically prepared asialo-rhuEPO, a non-erythropoietic recombinant human EPO derivative lacking sialic acid, has been reported to display remarkable anti-apoptotic and

tissue-protective effects against damage caused by hypoxia, ischemia/reperfusion or cytotoxic agents in the brain, the heart, the kidneys and the liver. However, attempts to translate its protective effects into clinical practice is hampered by unavailability of suitable expression system and its costly and limited production from expensive mammalian cell-made erythropoietin (rhuEPO^{M}). We took advantage of a plant-based expression system lacking sialylating capacity but possessing an ability to synthesize complex *N*-glycans to produce a soluble, cytoprotective asialo-rhuEPO. We generated stable transgenic tobacco lines by co-expressing human *EPO* and β1,4-galactosyltransferase (*GalT*) genes to produce asialo-rhuEPO (Kittur et al., Plant Cell Rep., 2012, 31:1233-1243; PLoS One, 2013, 8: e764682013) and established its purification system (Kittur et al., Int. J. Biol. Macromolec., 2015, 72: 1111-1116; Plant Cell Rep. 2015, 34: 507-516). *In vitro* cytoprotection assays showed that the asialo-rhuEPO^{P} purified from transgenic plants provides better cytoprotection to neuronal-like cells (N2A) (Kittur et al., PLoS One, 2013, 8: e764682013), pancreatic β-cells (Arthur et al., Front. Pharmacol. 2017, 8: 208) and HL-1 murine cardiomyocytes (Kittur et al., unpublished data) from staurosporine-induced cell injury than rhuEPO^{M}. We also partly delineated its cytoprotective mechanisms. In STS-induced apoptotic pancreatic beta-cell model, we found that asialo-rhuEPO^{P} could suppress the activation of Mst1 as well as caspase-3, displaying its cytoprotective property, and that its anti-apoptotic effect could improve impaired insulin secretion through stabilizing PDX1, a key transcription factor involved in beta-cell development and function. In STS-induced apoptotic cardiomyocyte model, our study revealed that asialo-rhuEPO^{P}-mediated cardioprotection involves the suppression of Mst1 activation, thereby inhibiting apoptosis and restoring autophagy. Our findings set the stage for future *in vivo* cytoprotective studies in animal models of various diseases to determine whether asialo-rhuEPO^{P} could be developed as a broad cytoprotective agent. Currently, we are studying in vivo neuroprotective properties of asialo-rhuEPO in a mouse model of ischemia/reperfusion injury. These studies were supported by National Institute of General Medical Sciences grants (SC3GM088084; SC1GM111178-01A1) to J.H. Xie.

(166) GlycoNAVI: - GlycoAbun - Abundance Ratio of Glycans

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It has become clear that glycans are involved in various physiological functions. Since glycans of glycoprotein is biosynthesized by several enzymes in the process of post-translational modifications of proteins, there is a great diversity of glycan structures. Furthermore, glycan structures to be biosynthesized are affected by the environment. Therefore, the abundance ratio of glycans also changes depending on disease. Changes in glycan abundance ratios have been reported in glycoproteins, glycolipids and free glycans. Therefore, we are developing a database that stores these data from the literature.

This database, called GlycoAbun, stores information such as glycan structures, glycoconjugate structures, glycosylation sites, abundance ratios, related diseases (disease name, Disease Ontology, stage), and sample information (organism type, cell / tissue type, sex, age, etc.). These data are stored in Resource Description Framework (RDF) using the glycoconjugate ontology GlycoCoO and others.

In addition, this database is a resource of the glycoscience portal (GlyCosmos), developed under the support of JST/NBDC, and partners with GlyTouCan and the GlyComb glycoconjugate repository. Moreover, because we are collaborating internationally to develop GlycoCoO, we can easily collaborate through Semantic Web technologies with GlyGen, GlyConnect, and UniCarbKB, which all use the same GlycoCoO ontology.

(167) Next Generation Glycan Microarray Enabled by Next Generation Sequencing

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Glycans play important roles in many disease processes through specific interactions with other biomolecules such as glycan-binding proteins (GBPs). In the past decades, glycan microarray has become a standard tool for the analysis of ligand specificities for GBPs and a discovery platform for potential functions of GBPs. In a microarray experiment, a library of glycans are immobilized onto solid surfaces such as glass slides. The slides are interrogated with fluorescently tagged GBPs and the resulting fluorescent images represent specific bindings to certain glycan structures. Despite huge successes in its applications, there are several major challenges facing this technology. First, the numbers of glycans included in current glycan microarrays are limited by the amount of surface area on a microscope slide. With the current technology, it would be very challenging to incorporate more than 1000 glycans printed in replicates into one glycan microarray. Second, while glycan microarray is considered as a high-throughput platform due to the large number of glycans that can be analyzed simultaneously, it actually suffers from a bottleneck in processing that requires a manual alignment of a grid over the fluorescent image to quantify the fluorescent intensity at each individual spot. Thus, processing of many samples such as patient serum samples is a very labor intensive and slow process. Third, despite the simple concept, microarray technology is limited to a number of very specialized laboratories due to the high cost of instrumentation including microarray printer and scanner. To address these challenges, we have developed Next Generation Glycan

Microarray (NGGM) enabled by Next Generation Sequencing (NGS). In this new approach, each unique glycan is coded by covalently attaching a unique oligonucleotide sequence using click chemistry. These coded glycans are mixed in a single well of a 96-well plate and individual wells containing the coded glycans are interrogated with a single GBP attached to magnetic beads. DNA coded glycans bound to the GBP are pulled down and washed to remove unbound glycans by magnetic separation techniques. The DNA codes in the pulldown product are amplified by PCR and sequenced using NGS. The copy number of each code represents the relative binding affinity of the corresponding glycan. We validated this platform with a library containing 48 glycan-DNA conjugates and GBPs including 9 lectins and 4 antibodies. Our data showed that NGGM revealed similar patterns of glycan-GBP binding when compared to a slide-based glycan microarray with the same glycans. NGGM can provide a higher throughput process with higher sensitivity, greater dynamic range, and more versatile applications. For example, mammalian cells and bacteria are very difficult to analyze in the current glycan microarray format, but with the NGGM this may be a relatively simple process.

(168) Plasma Membrane-Associated Neu3 Sialidase Controls A Mechanism of Host Protection Against Chronic Intestinal Inflammation

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Recent published discoveries from this laboratory include the identification of recurrent low-titer non-lethal gastric infections of Gram-negative *Salmonella enterica* Typhimurium (*ST*), a major source of human food poisoning, as a cause of chronic inflammation of murine intestinal tissue, predominantly the colon, which persisted following pathogen clearance and escalated in severity with repeated infections. The resulting colitis may represent an origin of human Inflammatory Bowel Disease including Ulcerative Colitis. In the model of human food poisoning that we developed, we found that disease onset was linked to the disruption of a previously unknown mechanism of host protection resulting from the induction of intestinal neuraminidase (Neu) activity. The induction of Neu activity was closely associated with increased Neu3 expression. Increased Neu activity accelerated the molecular aging and clearance of host enterocyte intestinal alkaline phosphatase (IAP) by the de-sialylation of nascent IAP at the cell surface. This finding reveals that glycan remodeling is also involved in protein aging and turnover at the cell surface. We have investigated the role of Neu3 in the origin of colitis caused by recurrent low-titer bacterial infections that model human food poisoning. Our findings using Neu3-deficient mice indicate that Neu3 is required for the onset and progression of colitis. Absence of Neu3 resulted in a significant reduction of disease linked to maintenance of normal IAP levels, sufficient to detoxify the endotoxin lipopolysaccharide (LPS) produced by various commensal bacteria in the colon. In addition, we observed that intestinal Mucin2, a major component of the protective barrier of the colon, was also de-sialylated and decreased in expression during disease progression linked with increased inflammation. Neu3 deficiency maintained normal Mucin2 protein levels coincident with protection from colitis. These results indicate that intestinal Neu3 expression controls the rate of glycoprotein de-sialylation in a critical role that supports both IAP and Mucin2 functions, and acts as a gateway in the pathogenesis of chronic intestinal inflammation.

(169) SULF2 expression is prognostic for overall survival in Head and Neck Squamous Cell Carcinoma

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Head and Neck Squamous Cell Carcinoma (HNSCC), the sixth leading cancer worldwide, arises from oral cavity, laryngeal, and hypopharyngeal regions. The five-year overall survival rate of HNSCC patients is about 40% and poor outcomes are due to the lack of suitable markers for the detection of the cancers at an early stage with favorable prognosis and clinical outcomes.

Sulfatase 2 (SULF2) is an extracellular endosulfatase that post-synthetically edits the sulfation pattern of heparan sulfate proteoglycan (HSPG) via removal of 6-O-sulfate group from the heparan sulfate chains. SULF2 modulates the affinity of heparan sulfates on HSPG for growth factors thereby influencing downstream activity of the signaling pathways. Implicated as a driver of carcinogenesis, SULF2 promotes growth and metastasis of solid tumors. Here we explored the relationship between *SULF2* expression and clinical outcomes of HNSCC patients on both mRNA and protein level.

Differential expression analysis using RNA-seq data from NCI Genomic Data Commons revealed that *SULF2* mRNA is 2.2-fold higher in tumor compared with paired normal samples

($p < 0.0001$). One-way ANOVA showed that *SULF2* mRNA does not differ by tumor stage but is differentially expressed among the HNSCC tumor locations. HNSCC associated with human papillomavirus infection differs from HNSCC caused by tobacco and alcohol consumption in molecular characteristics and is associated with better clinical outcomes. We found that *SULF2* expression in HPV-negative patients is 2.17-fold higher than in HPV-positive patients, whose *SULF2* expression is the same as in normal samples. Most importantly, high *SULF2* mRNA is significantly correlated to poor overall survival of HNSCC patient (hazard ratio = 1.49, $p < 0.01$) after adjusting for age, gender, clinical stage and tumor location.

Immunohistochemistry staining of SULF2 protein in a cohort of 124 HNSCC patients with tumor located in the oral cavity detected SULF2 in 80% of the specimens (100 out of 124). Our analysis shows that strong SULF2 staining is correlated with poor survival in the HNSCC patients (hazard ratio = 1.65, $p = 0.198$) after adjusting of age, gender and stage. The trend is not significant due to limited sample size and semi-quantitative nature of the IHC staining but is consistent with the results of the genomic study.

In conclusion, we found that overexpression of*SULF2*in HNSCC on the mRNA and protein levels correlates with poor overall survival. We now follow the findings with examination of the heparan sulfation and HSPG carries with the expectation that they will serve as biomarkers for the detection and prognosis of HNSCC.

(170) In vivo tropism of Salmonella Typhi toxin to cells expressing a multiantennal glycan receptor

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Typhoid fever is a life-threatening disease, but little is known about the molecular bases for its unique clinical presentation. Typhoid toxin, a unique virulence factor of *Salmonella* Typhi (the cause of typhoid fever), recapitulates in an animal model many symptoms of typhoid fever. Typhoid toxin binding to its glycan receptor Neu5Ac is central, but, due to the ubiquity of Neu5Ac, how typhoid toxin causes specific symptoms remains elusive. Here we show that typhoid toxin displays in vivo tropism to cells expressing multiantennal glycoprotein receptors, particularly on endothelial cells of arterioles in the brain and immune cells, which is in line with typhoid symptoms. Neu5Ac displayed by multiantennal N-glycans, rather than a single Neu5Ac, appears to serve as the high-affinity receptor, as typhoid toxin possesses five identical binding pockets per toxin. Human counterparts also express the multiantennal Neu5Ac receptor. Cumulatively, these results reveal remarkable features describing how a bacterial exotoxin induces virulence exclusively in specific cells at the organismal level.

(171) Endothelial heparan sulfate is anti-thrombotic in vivo

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Heparan sulfate (HS), a highly sulfated polysaccharide, abundantly expresses in the vasculature. Anticoagulant HS (HS^{AT}) with the unique high affinity antithrombin (AT) binding pentasaccharide motif, was believed to be responsible for nonthrombogenic property of endothelium. However HS3st KO mice, which lack of the critical AT binding structure, don't have aprocoagulantphenotype. It indicates that normal level of$HS^{AT}$is not essential for homeostasis. Our researchexaminedthe$Tie2Cre^{+}Ndst1^{fl/fl}$($Ndst1^{CKO}$) mice in which the sulfate modifications on HS are highly reduced in both endothelial celland hematopoietic cellcompartments. $Ndst1^{CKO}$mice showed a shorter bleeding timein a tail bleeding test and a higher level of tissue fibrin deposition in lipopolysaccharide (LPS)-induced microthrombosis model, exhibiting a prothrombotic phenotype and documenting the*in vivo*evidence that HS functions to maintain hemostasis*in vivo*. By examining bone marrow transplanted mice, our study further determined that deficiency of endothelial HS, not the hematopoietic HS, led to the prothrombotic phenotype.Our study documented that endothelial HS is anti-thrombotic, and our ongoing study is proceeding to illuminate the underlying molecular mechanisms.

(172) Unraveling the activity of glycosyltransferases and other PTM enzymes with bioluminescent biochemical and cell-based assays

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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, glycosylation, and ubiquitination, and influence many aspects of normal cell biology and pathogenesis. Examples of post translational modification enzymes include but are not limited to, kinases/phosphatases, methyltransferases/demethylases, and glycosyltransferases/glucanases. Under normal physiological conditions, the regulation of PTM enzymes is tightly regulated. However, under pathological conditions, these enzyme activities can be dysregulated, and the disruption of the intracellular networks they govern 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. Current assays to analyze cellular or biochemical PTM enzyme activities can

be tedious, non-homogeneous, and not easily adaptable to HTS. They can also suffer from generation of false hits due to compound interference, or require special instruments for detection. To overcome these shortcomings, we developed an array of biochemical and cellular bioluminescent assays to detect these enzyme activities. This presentation will showcase how cellular mechanisms driven by PTM in general and phosphorylation and glcnacylation in particular can be addressed by analyzing these enzyme's cellular activity using the NanoLuc Binary Technology (NanoBiT), a two-subunit complementation system based on NanoLuc luciferase. We will also discuss how the bioluminescent nucleotide assays that detect UDP, GDP, UMP and CMP as means to measure glycosyltransferase activities can streamline studies on GT specificity of transfer of different sugars to different acceptors. We will show their use in screening for specific inhibitors as well as in studies of their modes of action. Development of these bioluminescent detection assays will enable the investigation of key signaling pathways and the study of a large number of PTM enzymes in general and GTs in particular, which may have significant impact on diverse areas of glycobiology research.

(173) Comprehensive Analysis of N-glycans of Serum IgG from the Ferret-an Animal Model for Human Influenza Virus Infection

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Influenza virus infection continues to be a major health problem and poses a major challenge to our community. To better understand influenza pathogenesis and virus transmission, a relevant animal model is important. Because of its human-like respiratory system, the ferret among many experimental animals is the most used model organism to study influenza pathogenesis and transmission, as well as to evaluate virus antigenic changes for influenza vaccine strain selections. The humoral immune response, consisting of influenza-specific IgGs is one of the major protective mechanisms against influenza viruses. Yet, whether ferret IgG is N-glycosylated, and if so, the nature of the N-glycan structures, and ultimately, what role these N-glycans play in ferret IgG-mediated protection against influenza viruses have not yet been investigated. Here we show for the first time that IgGs isolated from naïve and influenza-infected ferret sera are N-glycosylated, and that the N-glycan structures are complex and diverse. After being released from purified ferret IgGs by PNGase F, the N-glycans were labeled either through permethylation after reduction and then analyzed by MALDI-TOF/MS and LC-MS/MS, or RapiFluor-MS tag to assist chromatographic separation and detection by fluorescence and then analyzed by UPLC and LC-MS/MS. With the mass information (m/z) and fragmentation patterns, plus assistance from the exo-glycosidase treatments, 42 of N-glycan structures were assigned. Results showed that, in general, N-glycans of ferret IgG were similar to those of human IgG. They contained mainly complex-type of glycans, and some high-mannose and hybrid glycans. The complex-type glycans were primarily bi-antennary, both core fucosylated and non-fucosylated, and either with or without bisecting GlcNAc. A minor fraction of the glycans carried either mono-, or di- a2,6Neu5Ac(s). Moreover, Galα1-3Gal epitope was identified on the a1,6Man arm of three mono-sialylated and one nonsialylated N-glycans in ferret IgG, which is the major difference from human IgG. Importantly, changes in N-glycan profiles of serum IgG from ferrets infected with influenza A were observed, mainly in an increase of N-glycans with bisecting GlcNAc and F1A2G0, and a corresponding decrease of F1A2G1, suggesting that the glycosylation of ferret IgG may play some role in its functionality. These results underscore the relevance and cruciality of the ferret model for studying human influenza viruses.

(174) The GlyCosmos Web Portal: glycan structures, glycogenes, glycoproteins, pathways, diseases and more!

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The Life Science Database Integration Program led by the Japan Science and Technology (JST) and National Bioscience Database Center (NBDC) has been sponsoring the integration of life science databases for over ten years. As a part of this program, most recently, the international glycan structure repository GlyTouCan was developed to fill the gap of the lack of a repository for glycans, and it now allows scientists to register glycans and obtain accession numbers for use in publications. These accession numbers also allow glycan databases, as GlyTouCan Partners, to easily link with each other, and GlyTouCan provides these links from each glycan entry page.

The remaining gap to be filled was that between the glycosciences and other omics fields, especially genomics and proteomics, which are closely related to glycans. Considering that glycan function is very closely related to the genes that synthesize them as well as the proteins that they decorate, it was important to integrate these datasets more closely. Thus, new funds were recently granted to develop a Glycoscience Portal, which we call GlyCosmos. Now in the second year, GlyCosmos provides a web interface to not only learn about glycans, but also to learn about their related genes, proteins and lipids. Since the PDBj and jPOSTrepo are also funded by this program, collaborations are now in place to link with protein structures and proteomics data, respectively. GlyCosmos will also provide access to not only GlyTouCan, but also a new Glycoconjugate Structure Repository, called GlyComb, and a new Lipid Structure Repository. GlyCosmos will also include a pathway database for understanding glycan function, and a Total Glycome Database which is a database of glycomes of various types of cells and cell lines. Integration is also being planned with the U.S. GlyGen project, GlyConnect as well as UniCarbKB. Thus in the near future, users should be able to get a grasp of glycan structures and their functions just by accessing GlyCosmos Portal.

(175) Cell based engineering for production of recombinant GAG core proteins

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Glycosaminoglycans (GAGs) are *O*-linked glycans initiated by the formation of a conserved tetrasaccharide linker region and are further extended by unique classes of disaccharide repeat structures. Glycoproteins carrying one or more GAG chains are called proteoglycans, and can be found as secreted, transmembrane, or glycosylphosphatidiylinositol (GPI)-anchored proteins. The linker regions of GAG core proteins are extended as either heparan sulfate (HS) or a chondroitin sulfate (CS) disaccharide repeats and subsequently subjected to further modification by epimerization and sulfation. The type of GAG chain extended from the linker tetrasaccharide is determined by the combination of the structure of the underlying core protein, modifications occurring to the linker tetrasaccharide, the availability of the respective GAG chain biosynthetic enzymes, and the biochemical environment of the Golgi apparatus, where GAG polymerization, sulfation, and epimerization take place.

The enzymes involved in GAG extension are commonly hetero-oligomeric complexes in the Golgi apparatus. A co-complex of EXT1 and EXT2 is critical for HS polymerization, whereas varied combinations of CSGALNACT1, CSGALNACT2, CHSY1, CHPF, and CHSY3 are responsible for CS/DS polymerization. In order to generate engineered GAG chains on recombinant core proteins we employed CRISPR-Cas9 gene editing approaches to generate knock out cell lines deficient in HS or CS extension. Wild type HEK293 cells produced both HS and CS chains on recombinant Glypican-1 and Syndecan-1, while EXT1 KO cell lines showed the presence of only CS on each GAG core protein tested. In contrast, recombinant Bikunin contained CS GAG chains in both cell lines, but increased content of CS chains (2.5-fold) were observed for several GAG core proteins produced in the EXT1 KO cell line. In contrast, cell lines harboring a double disruption in CSGALNACT1/CSGALNACT2 led to a drastic decrease in the CS content on recombinant GAG core proteins compared to WT cells, but did not eliminate CS completely. Cell lines harboring the triple disruption in CHSY1/CHPF/CHSY3 led to complete elimination of CS on recombinant Bikunin.

Thus, our results indicate that the EXT1 KO cell line is defective in HS biosynthesis and results in enhanced CS production on GAG core proteins. Cell lines harboring combinations of CS co-polymerase gene disruptions lead to reduced CS extension, but only the triple KO of CHSY1/CHPF/CHSY3 led to complete elimination of CS content on recombinant GAG core proteins. In conclusion, we have initiated studies to generate glyco-engineered cell lines capable of synthesizing remodeled GAG chains on recombinant core proteins as tools to study the roles of these proteoglycan structures in biological systems. (supported by NIH grant P41GM103390).

(176) Identification of C-mannosylated proteins in human pluripotent stem cells by secretomics

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C-mannosylation is a poorly studied type of protein glycosylation characterised by the attachment of a single mannose to tryptophan residues within the consensus sequence WXXW/C. C-mannosylation sites are highly conserved in type I cytokine receptors and secreted proteins containing thrombospondin type 1 repeats (TSRs) but can also be found in many unrelated proteins. The modification affects protein function, folding or secretion. In mammalian cells, two C-mannosyltransferases (C-manTs), named *DPY19L1* and *DPY19L3*, with distinct specificities regarding the consensus sequence are known (1). We observed that C-mannosylated peptides are highly underrepresented in shotgun proteomic approaches which might explain why only less than 40 C-mannosylated proteins are known so far although bioinformatics predictions (2) suggest a much higher number.

Thus, as C-mannosylation is known to affect protein secretion, we decided to quantitatively compare levels of secreted proteins in wild-type (WT) and C-manT-deficient human

induced pluripotent stem cells (hiPSCs) in order to decipher the C-mannosylome of this cell type. Therefore, we deleted the C-manT genes *DPY19L1* and *DPY19L3* in hiPSCs using CRISPR-Cas and confirmed that the genomic deletions resulted in functional knock-outs (KOs) of the respective enzymes. Proteins from cell culture supernatants of WT, DPY19L1- and DPY19L3-KO hiPSCs were precipitated and analysed by LC-MS/MS. Label-free quantification (LFQ) revealed that 29 and 103 proteins were either absent or detected at significantly lower levels in DPY19L1- or DPY19L3-KO hiPSCs compared to WT cells. We applied a custom-made algorithm to identify proteins with consensus sites for C-mannosylation from our list of proteins that were less secreted in the C-manT-deficient hiPSCs and ended up with 41 proteins, including the already known C-mannosylated protein thrombospondin 1. The frequency of consensus sites for C-mannosylation was considerably increased among proteins with reduced secretion in C-manT KOs compared to all identified proteins, supporting our general approach. Currently, these consensus site-containing proteins are recombinantly expressed at high levels enabling the direct proof of C-mannosylation by mass spectrometry. In addition, our list of putatively C-mannosylated proteins contains several cytokines influencing important developmental pathways and we will use our C-manT-deficient hiPSCs to study the role of C-mannosylation for the early embryonic development in future.

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(177) Probing Biological Roles of Tumor-Associated MUC1 with Chemistry Approaches

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Mucins of cancer cells exhibit malignant-associated alterations in their glycan composition. The increased expression and altered density of shorter glycoforms of mucin, such as *O*-linked *N*-acetylgalactosamine (Tn), sialic acid capped Tn (sTn), and Thomsen-Friedenreich (TF) antigen, are commonly observed changes in malignant and premalignant epithelia. The respective glycans are not only viewed as markers but also portray to have a functional role by serving as docking sites for endogenous receptors. These associations mediate tumor cell interactions, play a crucial role in tumor progression and also actively contribute to immune evasion. One of the main barriers to explaining the functional significance of glycan-based changes in cancer is the natural epitope heterogeneity found on the surface of cancer cells. To help address this knowledge gap, our research interests are focused on the preparation of novel synthetic tools to explore the role of tumor-associated glycans of MUC1 in the formation of metastasis *via* association with lectins. Our approach for further dissecting ligand-lectin interactions combines synthesis of a) structurally well-defined MUC1 glycopeptide models that allow for the control of the complexity of the chemical space of the multivalent ligands, and b) MUC1-derived glycopeptide positional scanning combinatorial libraries displaying native-like heterogeneous and aberrant tumor-associated *O*-glycan epitopes to examine epitope heterogeneity, the glycoside cluster effect, and steric hindrance effect of neighboring glycans on binding to lectins. A key component in the preparation of positional scanning libraries is determination of isokinetic ratios for near equimolar incorporation of each component of the mixture. The relative ratios of glycosylated amino acids necessary for equimolar coupling to the resin-bound amino acids were determined. Several methods for on-resin removal of acetyl protecting groups from glycopeptides libraries by base-catalyzed hydrolysis were evaluated. The use of ammonia in methanol was found the most effective, and was performed prior to TFA-promoted cleavage of glycopeptides from the resin. These novel chemical probes are attractive tools to elucidate the multivalent carbohydrate-lectin recognition processes at the molecular level.

(178) Enzymatic synthesis of teichoic acid-like capsule polymers from Gram-negative pathogens

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Group 2 capsule polymers represent crucial virulence factors of Gram-negative pathogenic bacteria. They are synthesized by enzymes called capsule polymerases. During the last decade, capsule polymerases have become attractive tools for the production of capsule polymers applied as antigens in glycoconjugate vaccine formulations. Conventional production of glycoconjugate vaccines requires the cultivation of the pathogen and thus highest biosafety standards, causing tremendous costs. With regard to animal husbandry, where vaccines could avoid the exuberant use of antibiotics, conventional production is not sufficiently cost-effective. In contrast, enzymatic synthesis of capsule polymers is pathogen-free, fast, offers high stereo- and regioselectivity and works with high efficacy.

In this study, we describe a new family of capsule polymerases that vastly increases the toolbox of enzymes available for biotechnological purposes. Its members combine glycosyltransferase and hexose-/polyol-phosphate transferase activity to generate complex poly(oligosaccharide phosphate) and poly(glycosylpolyol phosphate) polymers, the latter of which display similarity to wall teichoic acid (WTA), a cell wall component of Gram-positive bacteria. Using modelling and multiple sequence alignment, we show homology between the predicted polymerase domains and WTA type I

biosynthesis enzymes, creating a link between Gram-negative and Gram-positive cell wall biosynthesis. The polymerases of the new family are highly abundant and found in a variety of capsule expressing pathogens with both human and animal hosts like *Neisseria meningitidis*, *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Bibersteinia trehalosi* and *Escherichia coli*. Five representative candidates were purified, their activity confirmed using NMR spectroscopy, and their predicted folds validated by site-directed mutagenesis.

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(179) Synthetic galectin-3 oligomers as probes for glycan binding and biological activity

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Understanding how galectin-3 engages extracellular glycans to activate intracellular signaling events is central to deciphering its role as a modulator of cell behavior in healthy and pathological processes. However, establishing galectin-3 structure-function relationships is challenged by its dynamic assembly into oligomers with an ill-defined number of carbohydrate-recognition domains (CRDs). Here we will present synthetic galectin-3 oligomers as tools to study relationships between CRD valency, glycan binding, and extracellular signaling activity. Specifically, we create fusion proteins in which peptides that self-assemble into alpha-helical coiled-coils are fused to the N-terminus of galectin-3. By varying the amino acid composition of the coiled-coil forming peptide domain, we can create galectin-3 oligomers with 2-5 CRD domains. Using a construct having three CRD domains, we demonstrate that synthetic galectin-3 oligomers can recognize glycans on the surface of Jurkat T cells and induce phosphatidylserine exposure, similar to wild-type galectin-3. However, unlike wild-type galectin-3, trimeric galectin-3 oligomers do not induce Jurkat T cell agglutination, permeability to propidium iodide, or loss of metabolic activity, collectively suggesting weakened activity for activating pro-apoptotic signaling. Trimeric galectin-3 oligomers can crosslink the model glycoprotein, asialofetuin, into insoluble aggregates similar to wild-type galectin-3; however, the kinetics of aggregate formation and size of asialofetuin aggregates formed via trimeric galectin-3 oligomers are smaller than those formed via wild-type galectin-3. Together, these observations demonstrate that CRD valency determines the extent of glycoprotein crosslinking via galectin-3. Furthermore, these observations suggest that wild-type galectin-3 activity as an extracellular T cell signal may require a CRD valency greater than three, consistent with prior reports that galectin-3 can self-associate into pentameric oligomers. Moving forward, we envision that synthetic galectin-3 oligomers with user-defined CRD valency will establish key structural features that define galectin-3 activity as an extracellular signaling molecule, thereby identifying new opportunities for therapeutic intervention at the interface of galectin-glycan recognition.

(180) Engineering of glycosyltransferases for in-vivo glycan modification

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Immunoglobulin gamma (IgG) is the preeminent effector protein of immune system and is responsible for clearing of microbes. However, self-reacting IgG is responsible for a number of autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis. Intriguingly, IgG exhibits well known paradoxical anti-inflammatory activity, in the form of high-dose intravenous immunoglobulin (IVIG). In fact, many inflammatory and autoimmune diseases are treated by high-dose IVIG. Studies by a number of groups over the last decade have shown that the glycan composition at asparagine-297 (N297) residue on each heavy chain of IgG dictates anti-inflammatory activity. In particular, terminal sialic acid has been shown to reduce binding of IgG to type I Fc-gamma-receptors, thereby reducing capacity to initiate ADCC and resulting in the anti-inflammatory function. However, creating hyper-sialylated IgG for therapeutic use is not trivial for a number of technical reasons. A number of reports have suggested that sialylation of IgG can occur extracellularly. Therefore, we examined whether sialylation could be added to IgG therapeutically. We have engineered two glycosyltransferases, namely b1,4-galactosyltransferase (B4GALT1) and a2,6 sialyltransferase (ST6GAL1), responsible for attaching galactose and sialic acid, respectively, on IgG glycan. These enzymes are type II membrane proteins canonically present in the *trans*-Golgi. We have fused the soluble, enzymatic domains of these enzymes to the Fc (Fragment crystallizable) of human IgG, in order to increase the stability and prolong the serum half-life in circulation. Proteolytic cleavage sites were removed to enable expression and production of full-length fusion proteins. The enzymatic transferase activity of both enzymes was confirmed *in vitro*. Further, these Fc-fusion glycoenzymes, when administered to mice, were able to increase terminal sialylation on IgG and attenuate arthritogenic serum-induced arthritis and nephrotoxic-nephritis models. Thus, soluble glycosylation enzymes represent a novel therapeutic approach for conditions in which benefit from modulating glycans.

(181) The cellular impact of glycoengineering

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Protein glycosylation is fundamentally important to most biological processes and it is often important to regulate it in biopharmaceutical development. Thus, substantial efforts have been taken to engineer glycosylation of a variety of biologics. However, the diversity and complexity of glycosylation have made it difficult to control glycosylation and unravel how engineering efforts impact the host cells. To address this challenge, we have comprehensively studied the impact of glycoengineering on 166 CHO cell clones, consisting of a wide array of single and multiple glycosyltransferase knockouts. First, we utilized a novel computational platform to study the changes in glycosylation seen in each mutant. Through this we identified dominant glycosyltransferses in CHO cells, and studied instances wherein the cells differentially expressed isozymes in response to a knockout. Second, we demonstrate how different knockouts resulted in changes in CHO cell phenotypes of interest to bioprocessing, including cell size, growth, viability, and metabolism. Finally, we conducted a large-scale RNA-Seq study of the clones, and found specific pathways were perturbed when the MGAT and other enzyme families were knocked out. Through this effort we are gaining a more comprehensive view of the molecular and phenotypic impact of glycoengineering on biopharmaceuticals and the host cell. We further provide a resource for future studies to understand how glycosylation is connected to diverse cellular processes.

(182) Development of Preparative Cora for Amplification and Preparation of Cellular O-Glycans

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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. In the CORA procedure, peracetylated benzyl-α-N-acetylgalactosamine [Bn-α-GalNAc$(Ac)_3$] is taken up by cells, deacetylated by cytosolic esterases to generate Bn-α-GalNAc which is transported into the Golgi apparatus. Bn-α-GalNAc mimics the O-glycan precursor GalNAc-α1-O-Ser/Thr (Tn antigen) and is subsequently elongated by the resident glycosyltransferases to form the Bn-O-glycans that correspond to the O-glycome of the cell. These Bn-O-glycans are secreted into the media and can be isolated and analyzed by MS to provide a profile of the O-glycome of the corresponding cell line. To determine if CORA can be used as amplify the O-glycome of a cell line, we needed to develop an O-glycan precursor that could function like Bn-α-GalNAc$(Ac)_3$, but be more sensitively detected and quantified. To address this, we prepared a 4-Azido-derivative of Bn-α-GalNAc (4-Azido-Bn-α-GalNAc), and demonstrated that it could serve as a substrate for the T-synthase. We then demonstrated that the peracetylated compound, 4-Azido-Bn-α-GalNAc$(Ac)_3$, when added to cells in culture, generated the 4-Azido-Bn-O-glycans in the conditioned medium that corresponded to the O-glycome of the cell line. Using the alkyne function of the fluorescent tag 2-amino-N-(prop-2-yn-1-yl)benzamide (PYAB) to conjugate it with the azido group of the complex mixture of 4-Azido-Bn-O-glycans, we demonstrated that the PYAB tagged O-glycans could be monitored during HPLC separation and purification. Using this approach with large cultures of A549 lung cancer cells, we have purified 70 mg PYAB labeled O-glycans from 4 L of conditioned medium. Based on the starting amount of 4-Azido-Bn-α-GalNAc$(Ac)_3$ and the amount of O-glycan generated based on UV absorption, the molar conversion rate from precursor to O-glycans was about 10%. Using MALDI MS analysis we have identified 34 PYAB tagged O-glycans with masses that corresponding to known O-glycans. We are currently using multidimensional HPLC to purify individual O-glycan derivatives as a tagged glycan library for printing on the NHS-derivatized glass slides to prepare the cellular O-glycome on A549 cells as a microarray. Thus, we have demonstrated that CORA technology is an efficient method to produce cellular O-glycans using cells as O-glycan factories.

(183) SimGlycan: Facilitating automated evaluation of glycoengineered therapeutic glycoproteins using MS methods

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Monoclonal antibody (mAb)- glycoproteins produced by living cell systems that are typically IgG1 molecules with N-Glycans attached to N297 residue on the Crystallizable Fragment (Fc) - based therapeutics are the fastest growing class of human pharmaceuticals [1]. Variation in IgG Fc glycoforms affects the safety, clinical efficacy, and effector functions of therapeutic antibodies including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) e.g., the terminal galactose enhances CDC activity, but fucose significantly decreases ADCC [2].

Hence, glycoengineering – cell engineering and chemoenzymatic engineering – is becoming a viable approach to produce mAbs with improved efficacy [2–4].

The IgG-Fc N-glycans possess more than 400 heterogeneous structures withvariable addition of fucose, bisecting GlcNAc, Gal, and sialic acid residues to the core G0, a complex biantennary heptasaccharide with monosaccharide composition GlcNAc2Man3GlcNAc2 [1,2]. Glycoengineering needs efficient qualitative and quantitative glycan analysis techniques for the development and quality control of therapeutic antibodies [2].

Mass spectrometry (MS)-based methods are used for analysis of therapeutic mAb Fc-glycosylation profiles [3,5]. Tandem MS enables identification of monosaccharide compositions and linkage orientation of the glycans based on glycosidic and cross-ring fragment ions [6] thereby enabling the quality evaluation of the glycoengineered mAbs e.g., monitoring specific transformation and change in relative quantities of a set of glycans between glycoengineered mAb vs the mAb produced in CHO cell lines [3].

Recently developed stable isotope labeling techniques -(1) mass shift e.g., 12[C6]-2-AA/13[C6]-2-AA [7], and (2) isobaric tags e.g., aminoxyTMT6, QUANTITY [8] - facilitate multiplexing experiments to be performed for the identification and quantitation of glycans by MS methods. The analysis of sialylated glycans by MS-based methods is challenging because of the differences in ionization efficiency between neutral and sialylated glycans that prevent direct quantitative comparison by their respective mass spectrometric signals. Recently developed integrated chemical strategy, Dual Reactions for Analytical Glycomics (DRAG) [9], enables quantitative comparison between neutral and sialylated glycans simultaneously using MS methods.

Efficient bioinformatics tools are required to facilitate evaluation of glycoengineered therapeutic mAbs using MS-based methods. We have created a database of N Glycan structures of therapeutic glycoproteins curated from published literature [1–5], develop new modules of SimGlycan software [10,11] to identify glycans using LCMS or MS templates, quantify by summing the LCMS peak areas of isotopologues and thus streamlining the evaluation of glycoengineered mAbs using MS methods.

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(184) Engineering agarose utilization into non-agarolytic Bacteroides thetaiotaomicron

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The microbiota of the distal gastrointestinal tract (GIT) of monogastric animals plays a vital role in maintaining host health, such as releasing energy and nutrients from dietary polysaccharides that are otherwise indigestible by human enzymes. These dietary polysaccharides can augment the beneficial properties associated with live bacteria in order to achieve an improved health outcome, which is known as synbiotics. Agarose is a marine polysaccharide that is well suited to act as a selective nutrient in a designer synbiotic system because it is resistant to digestion by the vast majority of microorganisms residing in the GIT of terrestrial animals. The chemical structure of agarose consists of repeating disaccharide subunits of 4-*O*-α-3,6-anhydro-L-galactose (AHG) and 3-*O*-β-D-galactose (GAL). Agarose can be completely saccharified into its monosaccharide substituents by three agarases found in a polysaccharide utilization loci (PUL) from a human intestinal bacterium *Bacteroides uniformis* NP1. This project aims to engineer an agarolytic strain of the commensal gut bacteria, *Bacteroides thetaiotaomicron* (*B. theta*), by introducing intrachromasomal copies of the three agarases engineered with secretion peptides that traffick protein to the extracellular surface. The transgenic agarases will be displayed on the outside of the cell in order to provide the enzymes access to agarose substrates, and growth will be contingent upon the import of released GAL through intrinsic GAL transporters. The engineered agarolytic strain is able to completely saccharify the complex carbohydrate and is well-suited to grow in the presence of agarose. This engineered strain can serve as a platform to study positive selection within the intestinal environment. Furthermore, engineering agarolysis into *B. theta* will serve as the first step in developing a formulated delivery system that could enable the targeted release of bioactive molecules co-administered with agarose.

(185) Synthetic glycan analog libraries for the development of selective, high affinity ligands of siglecs and galectins

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The siglecs and galectins are families of glycan binding proteins (GBPs) that are involved in a diversity of physiological and pathological processes, including infectious diseases, cancer, autoimmune diseases, allergies, Alzheimer's disease and numerous other diseases. These processes are mediated through binding of the carbohydrate recognition domain (CRD) or glycan binding site of these GBPs to glycan epitopes present on *N*- and *O*-linked glycoproteins, and glycolipids. For instance, galectins and siglecs commonly bind to LacNAc and sialyl LacNAc containing glycans, respectively. Though in many cases single-site binding affinities to 'natural' glycans are low (mM-mM range), multivalent interactions between multiple CRDs and glycans can yield functional high avidity binding *in vivo*. High affinity and selective ligands of these GBPs that can block their interactions with glycans *in vitro* or *in vivo* would be valuable tools for investigating their functions or serve as potential therapeutics. A productive strategy to develop selective, high affinity monovalent ligands has been based on glycan analogs which use privileged glycan scaffolds modified with non-carbohydrate substituents to increase affinity and selectivity. To develop ligands of galectins and siglecs, we have chemo-enzymatically synthesized libraries of glycan analogs using LacNAc and sialyl LacNAc scaffolds, respectively. We use a combinatorial approach to synthesize analogs based on sulfonamide, urea, thiourea, 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).

(186) Synthesis of structurally homogeneous heparan sulfate oligosaccharides for biological studies

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Glycan Therapeutics LLC

Glycan Therapeutics is a pioneer in the commercial synthesis of structurally defined heparan sulfate and heparin oligosaccharides. Our rapidly growing catalog contains more than 150 high purity oligosaccharides. The oligosaccharides are 6-18 sugars long and their structures are confirmed by NMR and MS. Different oligosaccharide derivatives are also available, including UV-labeled oligosaccharides (PNP), clickable oligosaccharides (Azidamido), affinity tagged oligosaccharides (Biotin) or fluorescent-labeled oligosaccharides (Fluorescein tag). Our compounds have been successfully used in a variety of applications in investigating the binding of heparan sulfate and proteins, including biolayer interferometry, heparan microarray, real time binding assays, and complex for co-crystals. Our products have been used in fields of research including neuroscience, anti-coagulation, inflammation, and viral/bacterial infections. At Glycan Therapeutics, our goal is to provide reliable, affordable, and pure heparan sulfate oligosaccharides to advance biological research involving heparin and heparan sulfate.

(187) Chemo-enzymatic Synthesis of the Functional Core M3 O-Mannose Glycan on α-Dystroglycan

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α-Dystroglycan is a peripheral membrane protein that is a key component of the "dystrophin-glycoprotein complex" that is essential in anchoring cells to the extracellular matrix (ECM) of tissues. Due to its significance as part of the bridge between extracellular matrix and the cytoskeleton of cells, α-dystroglycan plays a major role in the architecture and integrity of many human tissues. Disruption of the functionality of this primary link consequently plays a role in a number of very serious pathologies including congenital muscular dystrophies. The ligand for extracellular matrix receptors presented by α-dystroglycan is a fully elaborated "core M3" O-mannose glycan that appears to be unique to α-dystroglycan. In the past decade, our group, in collaboration and competition with other groups, has elucidated the underlying genes, their encoded enzymes, and the glycan structure built that is responsible for this crucial binding to ECM proteins. In this recent work, we are recapitulating each elongation step in vitro with the ultimate goal of being able to synthesize a fully elaborated core M3 O-mannose glycopeptide. Intermediate glycopeptides are being utilized to raise antibodies for further studies of normal and pathological tissues and cell lines. Based on these efforts, we will present data on the kinetics of the enzymes POMGNT2, B3GALNT2 and POMK (a member of a recently recognized class of kinases) as well as describe the utility of our newly developed core M3 antibodies for interrogating cell lines and tissues. This work was supported in part from a grant from NIH/NIGMS (LW and DL, R01GM111939).

(188) An enrichment strategy for analysis of N- and O-glycoproteome through mass spectrometry

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Glycosylation occurring in both prokaryotes and eukaryotes is a highly micro-heterogeneous post-translational modification (PTM) on proteins and lipids. Identification and detailed characterization of protein-linked glycans are important to elucidate their biological roles and use them as disease biomarkers. High-resolution mass spectrometry coupled with tandem MS^n

analysis is the most powerful method for the characterization of protein glycosylation. Low abundance and microheterogeneity make it difficult to elucidate glycosylation of proteins expressed at low levels. The typically poor ionization efficiency of glycopeptides further complicates the analysis. Enrichment of glycosylated fraction is a promising approach to maximize the detection of glycopeptides and their glycoforms. Here, we report a multistage enrichment strategy for glycoproteins using cellular fractionation and subsequent ZIC-HILIC solid-phase extraction of glycopeptides. Beside enabling the detection of low abundant glycoforms, our method is unique in that it is also capable of detecting glycopeptides bearing metabolically incorporated non-natural monosaccharides without extensive chemical tagging procedures. Moreover, our method makes it possible to localize non-natural sugars on the glycan termini. Thus, this approach allowed us to simultaneously assign both natural glycan bearing glycopeptides and metabolic incorporated ones with the non-natural form of sialic acids (via ManNAc), GalNAc and GlcNAc on human T-lymphoma (Jurkat), MCF-7 and PC-3 cells. This strategy has great potential for the discovery of novel biomarkers for pathological conditions by the unambiguous characterization of minor glycoforms of glycoproteins and also the dynamic glycosylation changes study through the metabolic installation of non-natural sugars.

(189) Glycan function independent of glycoprotein identity: Cell Surface Glycan Engineering reveals that matriglycan rescues Lassa Virus infection in dystroglycan-deficient cells

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Lassa virus (LASV) is a pathogenic infectious agent in humans, responsible for causing Lassa hemorrhagic fever and killing 5,000 people annually. LASV enters cells via functionally glycosylated α-dystroglycan (α-DG) as the primary cellular receptor. α-DG is best known as an important cell surface glycoprotein essential for bridging the actin cytoskeleton and extracellular matrix (ECM). Functional glycosylation of α-DG requires over a dozen genes, initiated with *O*-linked mannose and subsequently elaborated in a series of glycosylation events, ultimately terminating with a glycosaminoglycan-like repeating disaccharide (-Xylα1,3-GlcAβ1,3-) named *matriglycan*. The matriglycan structure is the binding site for laminin globular (LG)-domain-containing ECM proteins and is required for LASV infection. To determine if LASV requires the aglycone (α-DG) for binding, internalization, and infection, we sought to engineer functional matriglycan on the cell surface of HAP1 human cells lacking α-DG. Using chemoenzymatic approaches, matriglycan of defined chain lengths was first immobilized on activated microarray glass slides. Matriglycan microarrays were generated to investigate direct binding characteristics of either the ECM protein Laminin-α1-(LG-4/5) or a widely used antibody which recognizes matriglycan, IIH6. These studies demonstrate that matriglycan alone, at particular degrees of polymerization, is sufficient for *in vitro* binding. To further test our hypothesis that the α-DG polypeptide is not required for infection, we generated bifunctional CMP-Neu5Ac derivatives that are modified at C-5 with different lengths of matriglycan and biotin as a tracking handle. ST6GAL1 can efficiently transfer the modified Neu5Ac molecules to both a model complex-type *N*-glycoprotein acceptor substrate, fetuin, as well as to glycoproteins on the surface of living cells. Remarkably, we demonstrate that LASV-pseudovirus infection can be restored by engineering matriglycan on sialic acid-containing glycoconjugates on HAP1 cells lacking either α-DG or the classical *O*-mannosylation pathway. In a competitive inhibition assay, matriglycan can inhibit LASV-pseudovirus infection into wildtype HAP1 cells at low micromolar concentrations. These results indicate that only matriglycan is necessary and sufficient for LASV-pseudovirus infection in the absence of α-DG. Our cell surface glyco-engineering studies emphasize the functional importance of post-translational glycosylation, independent of protein. This work was supported by NIH R01GM111939 (LW), P01GM107012 (LW, GJB), and P41GM103490 (LW).

(190) A new version of GlycanBuilder that handles a wider range of glycan structures

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GlyTouCan was initially released in 2015 as the international glycan structure repository. GlyTouCan uses WURCS as its base representation for glycans because existing formats were insufficient in their flexibility to uniquely and universally represent any and all glycans as published in the literature. Therefore, in order to obtain WURCS strings for existing or new glycan structures, conversion tools or glycan structure editors that can export WURCS needed to be developed. We focused on GlycanBuilder, looking for tools that can visualize glycan structures and convert glycan text formats. GlycanBuilder is a tool developed during the EUROCarbDB project and is utilized by researchers all over the world. This tool implements functionality to design glycan

structures via a graphical user interface and to convert glycans to arbitrary glycan text formats. It is also possible to input glycan text formats and visualize the glycan structures. However, until now, the monosaccharides and glycan structures that could be handled by GlycanBuilder were limited. Figures of glycan structures in GlycanBuilder could be displayed in a variety of formats such as the "Essentials" format, University of Oxford representation, etc. However, with the publication of the third edition of the Essentials of Glycobiology in 2016, a new Symbol Nomenclature for Glycans (SNFG) was proposed. SNFG extends the original format with the addition of several new monosaccharide symbols. From the above, we updated GlycanBuilder to include more visualization functionality, incorporate SNFG symbols, and provide WURCS conversion functionality.

As a result, we released GlycanBuilder2, which now supports the design of glycan structures having cross-linked substituents, nested repetitions, cyclic structures, and glycan compositions using symbols. In addition, probabilistic annotations can be assigned to glycosidic linkages and substituents. These glycan structures can be drawn using SNFG symbols now as well. With the implementation of WURCS conversion functionality, users can not only visualize glycan structures by inputting WURCS, but they can also obtain WURCS strings by drawing glycan structures on the GlycanBuilder canvas. Consequently, it is now possible to visualize glycans from any existing glycan text format supported by GlycanBuilder, such as GlycoCT, and convert it to WURCS. GlycanBuilder2 will thus allow users to acquire WURCS more easily, facilitating search and registration of glycan structures in GlyTouCan. This tool is available under an open source license at https://github.com/glycoinfo/GlycanBuilder2. Also, executable files are available at http://www.rings.t.soka.ac.jp/downloads.html.

(191) Glycoli: glycoengineering of novel pathways for protein N-glycosylation in the E. coli cytosol

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Metabolic engineering of*E. coli*has been highly successful in producing diverse free oligosaccharides for research and commercial applications. In this project we address the challenge to modify proteins in a site-specific manner with oligosaccharides. Starting point is a cytosolic N-glycosyltransferase (NGT) from*Actinobacillus pleuropneumoniae*, which transfers a single glucose residue onto proteins at asparagine (N) residues in the N-X-S/T sequon. Co-expression of an NGT with heterologous expressed bacterial glycosyltransferases has been shown to yield N-linked (poly)sialyllactose.

Here, we take this notion further by creating a modular glycoengineering toolbox, creating a system in which a great diversity of glycans can be created by the combination of glycosyltransferases. Glycosyltransferase biobricks (GTBb) were designed: each glycosyltransferase was imbedded in a tunable expression cassette carrying its own regulatory elements flanked by two regions harboring isocaudameric restriction sites. The resulting glycosyltransferase biobricks offer the potential for easy and endless assembly to build up artificial glycosylation pathways.

This system has lead to the generation of novel glycoproteins in the*E. coli*cytosol by modifying glycosylation sequons with a lactose primer that is further extended by different sialyl- and fucosyltransferases. This leads to the production of GM3, fucosyllactose and diverse (poly)sialyllactose structures on GFP.

To further enhance the power of this toolbox, we also engineered the target substrate. By creating multivalent glycosylation tags harboring several glycosylation sequons, multivalent glycosylation of proteins comes within reach.

Taken together, the Glycoli project provides a modular toolbox enabling the generation of a diverse repertoire of (multivalent) glycosylated proteins carrying epitopes relevant as reagents for fundamental research and as vaccine candidates.

(192) 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

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Cell surface or secreted sialoglycans that terminate in sialic acids (Sias) participate in and critically influence numerous and diverse biological processes, including cell-cell interactions, immune regulation, cellular homeostasis, host-pathogen interactions and microbial pathogenesis. Both mechanistic and discovery research on such important issues is impeded, not only because of the diverse structural complexities of the modifications and linkages of sialoglycans, but also because of inadequate techniques for detection. The full extent of sialoglycan diversity in nature is unlikely to be completely characterized in the near future, as conventional glycomic methods can destroy or overlook crucial aspects of Sia diversity, or fail to elucidate native sialoglycan structures as they exist in biological systems. Meanwhile methods for in situ detection and analysis of sialoglycans in situ ("the sialome") are limited to a few plant lectins, sialidase or antibodies whose specificities are limited and/or uncertain. Assuming that a simple and systematic approach to track sialome changes in a specific system would benefit researchers

even with little expertise in the field, we aimed to develop a set of recombinant and stably tagged Sialoglycan Recognizing Probes (SGRPs). We have defined 9 probe classes (SGRP1-SGRP9) targeted to the most predominant mammalian Sia types and linkages. Anticipating that the process of natural selection would have already maximized specificity/avidity during the rapid evolution of microbial molecules such as bacterial adhesins, toxin subunits and viral hemagglutinin-esterases, we selected the best candidates for SGRPs, each engineered with its corresponding non-binding mutant as a negative control. Binding specificity was first confirmed by a sialoglycan microarray displaying the commonly known structural diversity of common sialoglycans (with loss of binding of a control mutant for each probe), and the optimized panel of SGRPs was subsequently screened by common detection methods such as ELISA, Western Blot and FACS analysis, optimizing experimental conditions to minimize damage to relatively unstable Sia modifications such as O-acetylation. Comparisons were made with commonly used plant lectins. Our data demonstrate high order specificity of the SGRPs towards their target sialoglycans and results indicate that these probes could discriminate between structural differences. We also analyzed SGRPs by immunohistochemistry of mouse tissues including genetically modified strains. While much further work is needed to define a comprehensive suite of SGRPs, the current results will provide a simple and reliable toolkit to track the sialoglycome in mammalian systems, and we aim to demonstrate their implications in various physiological or pathological models.

(193) Chemoenzymatic Tools to "See" Sugar Epitopes on Cell Surfaces

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The major part of biological research related to glycoscience is to detect and quantify sugar epitopes in a variety of biological systems (such as on the cell surface, tissue surfaces, protein surfaces). Traditionally, the most important tool to "see" sugar epitopes is by the use of epitope specific antibodies and sugar lectins. However, specific antibodies to sugar epitopes are hard to generate, and currently, there are no specific antibodies for most of common sugar epitopes. In recent years, metabolic labeling strategy, by which bioorthogonal functional groups carried by unnatural monosaccharides analogues are metabolically incorporated into glycans *in vivo* for covalent conjugation by bioorthogonal chemical reactions to tag a specific class of glycans, emerged as a powerful tool for glycan analysis. Alternatively, chemoenzymatic labeling strategy provides an *in vitro* labeling method to detect structure-defined glycan epitopes. This method takes advantage of the relaxed donor substrate specificity and strict acceptor specificity of recombinant glycosyltransferase to label target epitope with bioorthogonal groups carried by unnatural nucleotide sugars. Recently, we have developed tools for sensitive, selective, and rapid analysis of Neu5Aca2,3 Gal glycan and sialyl-T glycan epitopes, respectively. Current method for the detection of Neu5Aca2,3 Gal glycan is based on lectin binding, which suffer from lower sensitivity and cross reactivity. Meanwhile, there is no direct detection method available for sialyl-T analysis. Therefore, the investigation of these two glycan epitopes has been very difficult. By chemoenzymatic labeling strategy, we successfully solved these problems.

(194) Lectenz® Affinity Reagents for Sialoglycoconjugate Detection and Enrichment

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Carbohydrate chains are often highly branched complex structures that pose great challenges for their detection, purification, and structural analysis. Advanced instrumentation such as mass spectrometry (MS) and high-throughput detection technologies such as lectin and antibody arrays have been developed to tackle such challenges. However, each technology has its limitations. For example, MS requires highly purified analytes and employment of complementary methods to solve linkage variation between glycan molecules that are of identical composition and mass. Anti-carbohydrate antibodies are not only difficult to generate due to a glycan's complex structural nature and poor immunogenicity, but are also often unable to broadly detect a glycan motif. Carbohydrate-binding proteins such as lectins of plant origin are limited by their source, purity, and varying specificity.

Using computationally-guided design and directed evolution, Lectenz® Bio is converting glycan-processing enzymes into catalytically inactive, high affinity glycan binding reagents with tunable specificities. These novel lectin-like enzyme-derived reagents called Lectenz® have been shown to offer numerous advantages over other carbohydrate-binding reagents: they are high affinity, yet retain the exquisite substrate specificity of the endogenous enzyme, they can be cost-effectively produced, and they can be used as capture/detection reagents in a variety of applications.

Here we report two novel sialic acid recognizing Lectenz® (patent-pending) engineered from a sialidase enzyme: 1) Sia-PS1, which recognizes sialic acid in a linkage independent manner; and 2) Sia-3S1, 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. Sia-PS1 has been shown to detect only sialylated structures on a glycan array and sialoglycoproteins on Western Blot. Similarly, we verified Sia-3S1's specificity for

Neu5Acα2,3Galβ1,4Glc, but not Neu5Acα2,6Galβ1,4Glc or Galβ1,4Glc. Both the pan-specific Sia-PS1 and the α2,3-linkage-specific Sia-3S1 have been used successfully in affinity chromatography for separation of sialoglycoproteins from nonsialylated glycoproteins.

(195) GlycoSense: A Rapid Method for Monitoring In Vitro Glycoengineering

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The use of enzymes to modify glycans, in order to generate uniform protein glycosylation for optimal bioactivity, or to test glycan-structure function hypotheses, or in chemoenzymatic synthesis, has emerged as a transformative technology. However, there are no convenient cost-effective methods for monitoring the progress of the enzyme reactions during *in vitro* glycoengineering.

Lectenz® Bio has developed an innovative technology called GlycoSense™ that will permit *in vitro* glycoenzyme reactions to be monitored in near real-time using flow cytometry and multiplex microspheres (beads). The GlycoSense™method detects binding between glycans and glycan-specific reagents that are conjugated to spectrally-unique beads. By combining the individual bead-based reagents into a multiplexed array, a profile of key glycan features can be obtained in less than a minute on a basic cytometer.

We illustrate the performance of the GlycoSense™ method using glycans and glycoprotein standards, and demonstrate its utility by monitoring changes in glycosylation patterns upon treatment with glycoenzymes. The GlycoSense™ approach will address the unmet need for a rapid tool to monitor protein glycosylation during *in vitro* glycoengineering.

(196) Benzylhydrosylamine (BHA) as a cleavable affinity tag for isolation and purification of reducing glycan for functional glycomics

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As one of the most important post-translational modifications, glycosylation occurs in over 50% of all human proteins and plays important roles in many biological processes including protein folding and stability, cell differentiation and adhesion, signal recognition and transmission, viral entry and bacterial infection, immune response and regulation. Currently a major obstacle for glycoscience study is the lack of diverse biomedically relevant, complex glycans in quantities sufficient for exploring functional aspects of glycan structure. Complementary to chemoenzymatic synthesis, natural glycans could serve as a great source of biomedically relevant glycans if they are available in sufficient quantities. We have developed oxidative release of natural glycans (ORNG) and optimized it for the rapid release of N-glycas as free reducing glycans. The capacity of this process is limited only by the size of the reaction vessel, making large quantities of free reducing glycans available to the research community. Due to the lack of a chromophore, a free reducing glycan cannot be easily monitored during chromatographic separation of a mixture from natural sources for either analytical or preparative purposes. However, free reducing glycans can be readily derivatized for HPLC and MS analysis, for glycoscience studies using popular tags such as 2-aminopyridine and anthranilic acid. While this is useful for analytical purposes, these tags are difficult to remove without affecting structural integrity of glycans if we wish to recover the glycans for preparative scale isolation. To address this inconvenience, we explored the use of a cleavable ultraviolet tag, O-benzylhydroxylamine (BHA). Free reducing glycans are easily and efficiently labeled with BHA under mild conditions, which show significantly improved HPLC profiles compared with unlabeled material. Individual glycan-BHA conjugates can then be separated using multidimensional HPLC and characterized by MALDI-TOF-MS and MS/MS. The BHA tag can then be easily removed by Pd/C catalyzed hydrogenation to efficiently regenerate free reducing glycans with little effect on glycan structures. This procedure provides a simple and straightforward way to tag large quantities of free reducing glycans for purification at a preparative scale using multidimensional preparative HPLC and subsequently recovering the purified free reducing glycans from the purified BHA conjugate for direct functional glycomics studies or for other derivatization procedures.

(197) Tick Bites and Hamburgers: N-Glycosylation analysis of saliva and salivary glands from the ticks responsible for Alpha-Gal Syndrome

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The CDC has reported that the number of new cases of disease transmission by mosquito, tick, and flea bites has more than tripled in the United States in recent years. The rise and risks of tick bites in particular has seen extensive coverage in many American news media outlets including the New York Times, NPR, CNN, and the Washington Post.

Amblyomma americanum, or Lone Star Ticks are found widely distributed in thesoutheastern United States but are rapidly expanding to more northeastern locations. Recent reports of allergic reactions associated with red meat consumption have been found among those bitten by Lone Star Ticks. This particular type of sensitivity was first reported during clinical trials of cetuximab, indicating the pre-

exposure and production of IgE antibodies specific for galactose-α-1,3-galactose. This alpha-gal hypersensitivity is now identified as a delayed-type food allergy to glycolipids and glycoproteins found in non-primate meat, induced following tick bites. This marks a rare occasion where the immune system is sensitized by a sugar rather than a protein, and has critical importance in the fields of immunology, as well as the pathology and epidemiology of tick-transmitted illnesses.

In this presentation saliva and salivary glands collected pre and post-feeding were obtained from *Amblyomma americanum* as well as other sympatric species (*Ixodes scapularis* and *Amblyomma maculatum*). The samples were homogenized and N-glycans were released, isolated, and permethylated for analysis. Mass spectrometry (NSI-FTMS) including fragmentation analysis by CID was used to identify each glycoform by mass and fragmentation patterns. Further determination of structure, including the presence of α-Gal, was also determined through MS/MS fragmentation analysis. These results provideextensive information regarding glycosylation versus tick species as well as feeding and nonfeeding glycosylation changes. Interestingly, indications of galactose-α-1,3-galactose were found in multiple species of tick, including those not implicated in the induction of alpha-gal hypersensitivity. Additional indications of aldopentose-decorated N-glycans were also observed, indicating that interactions between the tick outside of host feeding may have implications in tick-host transmission.

(198) UDP-Glycosyl or Glucuronosyl transferases in Caenorhabditis elegans: Insights into Roles in Xenobiotics Detoxification

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Uridine 5'-diphospho glucuronosyl or glycosyl transferases (UGTs) are involved in phase II xenobiotics metabolism, where they aid the detoxification of xenobiotics via glycosylation of small molecules. While some UGTs have been implicated in several transcriptomics studies when *Caenorhabditis elegans* are challenged with xenobiotics, there is very little knowledge of the modifications carried out by specific UGTs. In an effort to improve our knowledge of the roles of UGTs in *C. elegans*, we sort to identify glycosylation (UGT) genes involved in the detoxification of selected xenobiotics and their respective modifications.

In this light, we have developed a high-throughput toxicity and defective glycosylation assay (***H-TDGA***). *H-TDGA* incorporates a dead/alive toxicity assay using SYTOX green nucleic acid stain; and assays for defective glycosylation using High Performance Liquid Chromatography-Ultra Violet detector (HPLC-UV), High-Resolution Mass Spectrometry (HRMS), and Nuclear Magnetic Resonance (NMR) experiments, all for the detection of potentially glycosylated metabolites and structural elucidation. Using *H-TDGA*, we have exposed candidate UGT mutant lines to indole, a metabolite produced by *Escherichia coli*. Indole is toxic to *C. elegans* at high concentrations, and wild type animals are able to detoxify the xenobiotic via an *N*-linked glycosylation. Results of assays with UGT mutant lines will be presented.

(199) Synthesis of P1-(11-Phenoxyundecyl)-P2-(2-Acetamido-3-O-β-D-Galactopyranosyl-2-Deoxy-α-D-Galactopyranosyl) Diphosphate as an Acceptor Substrate for Bacterial Glycosyltransferases

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Bacterial polysaccharides have important functions in protecting bacteria from adverse environment and the host immune system. The lipopolysaccharides of Gram negative bacteria that function in bacterial survival and virulence contain O-antigenic polysaccharides on their outer membrane that are directed towards the environment. In *Escherichia coli* about two hundred different serotypes are known that have different O-antigenic polysaccharide structures. The assembly of these O-antigens is catalyzed by the specific glycosyltransferases and proceeds through the intermediate formation ofundecaprenyl diphosphate sugars as acceptor substrates. To study this process, a chemical synthesis of the compounds having the natural structure or their analogues is necessary. The phosphoroimidazolidate method is a universal method for the synthesis of lipid diphosphate sugars, particularly lipid diphosphate disaccharides containing a 2-acetamido-2-deoxyglycosyl residue at the reducing end of the disaccharide moiety and 11-phenoxyundecyl residue as the lipid moiety of the molecule. We have synthesized P^1-(11-phenoxyundecyl)-P^2- (2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-α-D-galactopyranosyl) diphosphate [Galβ1-3GalNAcα-OPO_3-PO_3-$(CH_2)_{11}$-O-phenyl] and verified its structure by TLC, NMR and MS. We used the compound as an acceptor substrate to assay bacterial enzymes involved in the biosynthesis of O antigen repeating units, i.e. purified α2,3-sialyltransferase WbwA from *Escherichia coli* serotype O104 and α1,4-Glc-transferase WclY from *Escherichia coli* O117. The chemical synthesis of purified acceptor compounds with defined structure facilitates the characterization of bacterial glycosyltransferases that could be targets to develop anti-bacterial compounds against pathogenic enteric bacteria.

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(200) Enabling tools for Toxoplasma glycobiology

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Infection by the protozoan parasite *Toxoplasma gondii* is a major health risk on account of its chronic nature, ability to reactivate to cause blindness and encephalitis, and high prevalence in human populations. Like nearly all eukaryotes, *Toxoplasma* glycosylates many of its proteins and lipids and assembles polysaccharides. While their structures resemble canonical models, they exhibit considerable species-specific variations that have inhibited deeper investigations into their roles in parasite biology and virulence. The genome of *Toxoplasma* encodes a suite of likely glycogenes that are expected to assemble a range of *N*-glycans, *O*-glycans, a *C*-glycan, GPI-anchors, and polysaccharides, along with their requisite precursors and membrane transporters. To facilitate genetic approaches to investigate the roles of specific glycans, we have mapped probable connections between 59 glycogenes, their enzyme products, and the glycans to which they contribute. We have adapted a double-CRISPR/Cas9 strategy and a mass spectrometry-based glycomics workflow to test these relationships, and conducted infection studies in fibroblast monolayers. Through the validated disruption of 17 glycogenes, we have identified known and novel *N*- and *O*-glycan structures on proteins in the nucleus or that are processed in the secretory pathway, mapped the contributions of many of the glycogenes to their assembly, and documented their evident significance for general growth. All guide sequences, disruption constructs, and mutant strains, are freely available to practitioners who can apply them in the context of the relational map to investigate roles of glycans in their favorite biological processes. The development of this toolbox is anticipated to open new avenues to explore glycan functions in *Toxoplasma*.

(201) Probing Specific Cell-Surface Heparan Sulfate-Protein Interactions

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Cell-surface heparan sulfate and its structurally related heparin bind a host of basic proteins that take advantage of the sugar's dense structural information. The significance of these interactions in various aspects of development, physiology, and disease stimulated keen interest in evaluating structure–activity relationships. The well-defined heparan sulfate and heparin oligosaccharides needed for these studies can be mainly accessed by chemical synthesis. The various synthetic strategies available to chemical synthesis have recently enabled the acquisition of several regular and irregular sequences through improved coupling methods and judicial protecting group manipulations. Investigations of various protein interactions with the synthetic constructs delivered valuable information that could aid future drug development endeavors.

(202) Preparation of an O-glycan Library from Porcine Stomach Mucin by Oxidative Release of Natural Glycans (ORNG)

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O-GalNAc glycosylation is the major post-translational modification of mucins and these O-glycans on mucin serve as ligands for various carbohydrate-binding proteins and mediate diverse biological processes. While a comprehensive library of these glycans is of great interest for their functional study, the access to these glycans through chemoenzymatic synthesis is laborious and limited to a number of specialized laboratories. On the other hand, certain natural materials such as porcine stomach mucin (PSM) could serve as abundant sources of diverse O-glycans. We have recently developed oxidative release of natural glycans (ORNG), which uses household bleach to efficiently degrade the amide linkages of the protein backbone and release O-glycans attached to a lactic acid (threonine) or glycolic acid (serine) as the major products (Song et al., 2016, 13(6): 528-34). The amount of starting material that can be processed by this method is dependent only on the size of the reaction vessel available. In this study, 50 grams of PSM were processed by the ORNG and the products were separated using size exclusion chromatography (SEC) and weak anion exchange (WAX) into free O-glycans, O-glycan glycolic/lactic acids and sulfated O-glycan acids. The major products, O-glycan acids, were tagged with monoFmoc ethylenediamine and separated by multidimensional HPLC using a combination of preparative C18 reversed phase HPLC and normal phase HPLC. A comprehensive library of PSM fluorescent tagged O-glycans was obtained and analyzed by mass spectrometry. Aliquots of the tagged glycan library will be treated with piperidne to reomove the FMOC making the library amenable to conjugation to solid supports including NHS-activated glass microscope slides for production of a shotgun glycan microarray of PSM for the studies of protein-glycan interactions.

Keywords: O-glycan; porcine stomach mucin; oxidative release; shotgun glycan microarray

(203) Enzymatic Synthesis of Homogeneous Chondroitin Sulfate E Oligosaccharides

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Chondroitin sulfate (CS) is a sulfated polysaccharide which contains repeating units of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc)→4)GlcAβ(1→3)GalNAcβ(1→The GalNAc residue can be modified by 4-*O*-sulfation (chondroitin sulfate A, CSA), 6-*O*-sulfation (chondroitin sulfate C, CSC) or 4,6-disulfation (chondroitin sulfate E, CSE). CSs are widely present on the mammalian cell surface and in the extracellular matrix to be involved in diverse physiological processes, *e.g.* chondroitin sulfate E was reported to be involved in neurite elongation, bone formation, interaction with midkines, and others. However, the CSs isolated from natural resources are present in a mixture of different sizes and different sulfated patterns. The lack of structural homogeneity is the major roadblock that hinders CS research. Recently, we reported an enzymatic method for synthesis of CSA and CSC oligosaccharides library with high efficiency (1). Base on this, we further synthesized structurally defined chondroitin sulfate E oligosaccharides ranging up to 19mer, and the scale can be up to hundreds mg scale. We also invested the biological functions of the chondroitin sulfate E 19mer. In a mouse model of histone-induced mortality, 80% of mice died within 20 minutes of infusion of 75 mg/kg histone H3. Pretreatment with 75 mg/kg CSE 19mer immediately before histone infusion protected mice from histone-induced death. Results from our studies offer a new tool to investigate the roles of chondroitin sulfate in biological processes.

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(204) Helicobacter pylori induces intracellular galectin-8 aggregation within gastric epithelial cells in both host O-glycan- and autophagy-dependent manners

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Galectin-8, a beta-galactoside-binding lectin, was previously reported to be upregulated in the gastric tissues of rhesus macaques infected by *Helicobacter pylori* (*H. pylori*). In this study, we found that *H. pylori* infection triggers intracellular galectin-8 aggregation in human-derived AGS gastric epithelial cells, and these aggregates colocalize with lysosomes. Notably, this aggregation phenomenon is markedly reduced following attenuation of host O-glycan processing. This observation indicates that *H. pylori* infection induces lysosomal damage, which in turn results in accumulation of cytosolic galectin-8 around damaged lysosomes through recognition of exposed vacuolar host O-glycans. *H. pylori*-induced galectin-8 aggregates also colocalize with autophagosomes, and galectin-8 ablation reduces *H. pylori*-mediated autophagy activation. This suggests a possible function of galectin-8 aggregates in enhancing autophagy activity in infected cells. We also observed that both autophagy and NDP52, an autophagy receptor, play a part in augmenting *H. pylori*-mediatedgalectin-8 aggregation. Besides, vacuolating cytotoxin A (VacA), a secreted *H. pylori* cytotoxin, may contribute to the increased galectin-8 aggregation and elevatedautophagy response in infected cells. Collectively, these results suggest that *H. pylori* promotes intracellular galectin-8 aggregation in both host O-glycan- and autophagy-dependent manners, and *H. pylori*-mediated galectin-8 aggregation and autophagy may reciprocally regulate each other during infection.

(205) Accelerated aging and clearance of anti-inflammatory enzymes in the pathogenesis of Gram-negative sepsis and its therapeutic reversal

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Sepsis is a life-threatening inflammatory syndrome accompanying an infection of the bloodstream. The leading cause of death in non-cardiac intensive care units, sepsis is increasing in incidence while no new effective therapies have been developed in decades. Frequently secondary to bacterial pathogens, sepsis remains difficult to treat as a singular disease mechanism. Experimental analysis of bacterial sepsis was undertaken in the murine model, where genomic signatures of inflammatory responses were recently shown to closely correlate to those of humans. For all bacterial strains, disease severity and mortality were directly proportional to increased bacterial cfu in the bloodstream. We thus applied criteria for data inclusion in comparative sepsis pathophysiology analyses involving attainment of minimum and maximum thresholds of blood cfu at specified times post-

infection. We compared the pathogenesis of sepsis elicited by five strains of bacterial pathogens and found similarities among host responses to Gram-negative *Salmonella* and *E. coli*. Our findings reveal a mechanism of host protection involving two circulating alkaline phosphatase (AP) isozymes that de-toxify the lipopolysaccharide endotoxin of Gram-negative bacteria. Sepsis caused by either *Salmonella* or *E. coli* incapacitated this protective mechanism by activating host responses to Toll-like receptor-4, resulting in the induction of Neu1 and Neu3 neuraminidases in circulation. Elevated neuraminidase activity increased the basal rate of molecular aging and clearance of both AP isozymes, similar to constitutive results observed in the absence of ST3Gal6 sialyltransferase deficiency, thereby intensifying disease. In contrast with studies of pneumococcal sepsis, deficiency of the hepatic endocytic Ashwell-Morell receptor was protective by reduced clearance of de-sialylated AP. In Gram-negative sepsis, AP augmentation or neuraminidase inhibition diminished inflammation and markedly increased the likelihood of host survival.

(206) Controlled expression of the periplasmic protein LmeA regulates the abundance of lipomannan and lipoarabinomannan in Mycobacterium smegmatis

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The mycobacterial cell envelope is a thick, multilayered complex composed of an outer membrane, an arabinogalactan-peptidoglycan cell wall, and a plasma membrane. Phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM) are phosphatidylinositol (PI)-anchored glycans, that are essential components of this cell envelope and modulate host immune response during infection. The biosynthesis of PIMs/LM/LAM begins by sequential additions of two mannoses and one fatty acid to a PI, resulting in the first product, AcPIM2. This PIM species also serves as a precursor for the biosynthesis of LM and LAM. An unknown α1,6-mannosyltransferase(s) elongates AcPIM2 to produce an LM intermediate, which is further elongated by another α1,6-mannosyltransferase, MptA. The α1,6-mannose backbone is additionally decorated by mono-mannose side chains mediated by the α1,2-mannosyltransferase, MptC. Recently, we reported a periplasmic protein, LmeA, which is involved in maturation of LM mannan chain in *Mycobacterium smegmatis*. Interestingly, a global secretome analysis indicated that the *M. tuberculosis* ortholog of LmeA is upregulated during mouse infection. However, the precise function of LmeA remains unknown. Therefore, in this study, we examined if changes in the expression level of LmeA has an impact on LM/LAM biosynthesis and cell envelope integrity. We established an *M. smegmatis* strain overexpressing the HA epitope-tagged LmeA (LmeA-HA OE) in addition to the previously established *lmeA* deletion mutant (Δ*lmeA)*, and an *lmeA-HA*-complemented Δ*lmeA* (Δ*lmeA*::*lmeA-HA*), in which plasmid-encoded *lmeA* is expressed from the native promoter. Remarkably, overexpression of LmeA-HA resulted in an increased abundance of both LM and LAM. There was no significant impact on the abundance of PIMs or other phospholipids, suggesting that this effect is specific to LM/LAM production. To start dissecting the mechanism, we examined if overexpression of LmeA-HA changes the protein levels of the known LM/LAM mannosyltransferases, MptA and MptC. We found that the level of MptA was significantly higher in the LmeA OE strains, while MptC abundance was relatively similar among LmeA OE, Δ*lmeA*, and Δ*lmeA*::*lmeA-HA* strains. Because the level of MptA is known to decrease in late stationary phase, we examined what impact the expression of LmeA would have on MptA under various conditions such as stationary phase, starvation, and hypoxia. We found that the abundance of MptA significantly decreased in the Δ*lmeA* mutant than in wild-type or LmeA OE under these stress conditions. Furthermore, LM was found more abundantly in LmeA OE under these conditions. Together, our current study reveals the impact of LmeA expression on the biosynthetic enzymes of LM/LAM, leading to changes in LM/LAM abundance. We are currently testing if such changes in the LmeA expression level have an impact on the cell envelope integrity.

(208) An essential mitochondrial fucosyltransferase in Leishmania parasites

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Recently, we characterized two *L. major* genes (*FKP40* and *AFKP80*)encoding bifunctional proteins with kinase/pyrophosphorylase activities (Guo *et al J. Biol. Chem.* 2017). Both could salvage L-fucose to GDP-L-Fuc, but only AFKP80 yielded GDP-D-Ara*p* from D-Arabinopyranose. Correspondingly, Δ*afkp80*-mutants lack D-Ara*p* while Δ*fkp40*-mutants resembled WT. Unexpectedly, we were unable to produce Δ*fkp40*-/Δ*afkp80*-double mutant. These data suggested that GDP-Fuc is essential.

The *Leishmania* genome predicts at least 5 candidate fucosyltransferases. Among them, Lm01.0100 is the only candidate FucT conserved amongst trypanosomatids, showing key motifs characteristic of a-1,2 fucosyltransferases. Enzymatic assays of *L. major* overexpressing C-terminally tagged Lm01 showed transfer of Fuc from GDP-Fuc to Galb1,3GlcNAc-β-O-Methyl. On this basis we renamed this gene Fucosyl Transferase 1 (*FUT1*). To test essentiality, we used a quantitative 'plasmid segregation' assay, expressing

FUT1 from the multicopy episomal pXNG vector, which additionally expresses GFP, in a chromosomal null Δ*fut1*⁻-background. Single cell sorting for GFP-null lines did not yield viable cells, indicating that *FUT1* is essential.

Unlike most eukaryotic glycosyltransferases, *FUT1* was predicted to localize to the parasite mitochondrion, which was confirmed by expression and imaging of a C-terminally tagged protein (FUT1-HA). To probe importance of mitochondrial localization, we used 'plasmid shuffling', asking whether pXNG-*FUT1* could be lost in the presence of mutated test *FUT1*gene. A minimal mitochondrial targeting sequence was identified, and this protein was fully functional. Modifications affecting mitochondrial localization could not rescue the Δ*fut1*⁻ mutant; each of these proteins was expressed at high levels but were now found in the cytosol. Mutation of the predicted FucT catalytic motif resulted in loss of function, while maintaining mitochondrial localization. Together these results suggest that both mitochondrial localization and FucT activity are essential for FUT1 functionality. Fortuitously, we were able to obtain a single rare Δ*fut1*⁻ mutant which displayed a severely decreased in growth rate and diverse defects in mitochondrial (kinetoplast) structure and function. Preliminary glycoproteomic analysis found many alterations in the mutant, as well as two proteins showing dHex modifications in WT but not Δ*fut1*⁻. Further analyses will determine if these proteins are potential FUT1 substrates.

Thus, *FUT1* joins the eukaryotic O-GlcNAc transferases as one of the first glycosyltransferases shown to act within the mitochondrion. Current work is now oriented towards identifying the *Leishmania* fucosylated targets therein, and the role of mitochondrial glycosylation.

(209) Post-CFG Glycan Microarrays and Their Applications

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Since first described by several groups in 2002, glycan microarrays have revolutionized the analysis of glycan-protein interactions, providing informative data of glycan-binding proteins (GBPs) in a high-throughput manner. Various glycan microarrays had been developed over the past 16 years,[1] among which the CFG glycan microarray that contains 600+ glycan structures unambiguously generated the majority of assay data, greatly facilitated the understanding of glycan-protein interactions. Nevertheless, focused glycan microarrays that contain a specific class of glycans are barely available. We are developing such post-CFG glycan microarrays and making them commercially available. For example, a library of 45 O-mannosyl glycans were chemoenzymatically prepared and spotted into a specific microarray, which revealed fine specificities of GBPs and antibodies.[2] In another example, we synthesized dozens of pairs of Neu5Ac/Neu5Gc containing glycans, which were used by researchers to identify novel Neu5Gc specific toxins/lectins. [3] Additionally, a microarray that contains 36 enzymatically prepared *N*-glycans demonstrated that internal epitopes and/or modifications of terminal epitopes could influence glycan-protein interactions. Other developed commercial glycan microarrays include N-glycan isomer array (100 N-glycans, manuscript in preparation), ganglioside array (58 structures), and human milk oligosaccharide array (46 structures), *etc*. We believe these focused microarrays will serve as ideal tools in understanding details of glycan-protein interactions, finding applications in various research areas.

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(210) Conservation of oxygen-dependent glycosylation in a plant pathogen, and implications for its dedication to the SCF ubiquitin ligase subunit Skp1

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Skp1 is hydroxylated by an oxygen-dependent prolyl hydroxylase (PhyA) that is postulated to be critical for oxygen-sensing in unicellular protists. In turn, glycosylation of the hydroxyproline is required to mediate, at least in part, the effect of prolyl hydroxylation. The mechanism involves an effect of the glycan on Skp1 conformation, which correlates with increased association of Skp1 with F-box proteins that serve as substrate receptors for E3(SCF)ubiquitin ligases. The glycan consists of five sugars, each of whose addition is mediated by a discrete cytoplasmically-localized sugar nucleotide-dependent glycosyltransferase (GT). In the social amoeba Dictyostelium, addition of the first sugar is mediated by a soluble alpha-GlcNAc-transferase (Gnt1), the second and third sugars are added by separate domains of the same processive di-GT (PgtA), and the fourth and fifth sugars are added by the same catalytic domain of a GT (AgtA) that possesses a separate Skp1-recognition domain. The agent for human toxoplasmosis, Toxoplasma gondii, conserves Gnt1 and PgtA but utilizes unrelated GTs for addition of the fourth (Glt1) and fifth (Gat1) sugars.

To further investigate the conservation and specificity of this cytoplasmic glycosylation pathway, we investigated related genes from the plant pathogen Pythium ultimum, an oomycete that causes damping off and root rot disease of hundreds of agriculturally important plants. Coding sequences for its PhyA and Gnt1 (the first GT) form a gene fusion that can complement a PhyA-deficiency in Dictyostelium, confirming a conserved function. Recombinant PhyA/Gnt1 is a Skp1 GlcNAc-transferase in vitro. This suggests the possibility of substrate channeling that

glycosylates all products of the prolyl hydroxylase reaction. In addition, it provides support for the model that PhyA serves solely to modify Skp1 because tritium-based biochemical complementation experiments using Dictyostelium and Toxoplasma Gnt1-KO extracts reveal only Skp1 as a Gnt1 substrate. Pythium has a homolog of Gat1, which we show to be structurally and functionally conserved with the fifth GT of Toxoplasma. Thus Gat1 is the primordial fifth enzyme, having been replaced by AgtA in amoebozoa en route to its evolving into the initiating glycogen GT glycogenin in yeast and animals. Overall, the data confirm the wide-spread distribution of this novel complex hydroxylation/glycosylation pathway in the cytoplasm of protists and further implicate Skp1 as its sole target. The prevalent fusion of Skp1 modification enzymes suggests that addition of only the odd-numbered sugars is regulated, because of anticipated processivity of the pairwise bi-functional enzymes that constitute the pathway in different protists.

(211) Analysis and recognition of anionic and zwitterionic glycans from invertebrates

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Recent data indicates that the diversity of N-glycan modifications in non-vertebrate species is extremely high. From our own studies on *Dictyostelium*, *Trichomonas*, *Penicillium*, molluscs, insects (whether mosquitoes, moths or the honeybee) and nematodes (including*Caenorhabditis)*, 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, phosphoethanolamineand phosphorylcholine. Here we summarise recent glycomic data on N-glycans from insect and nematode sources as well as selectedarray data on natural oligosaccharides from such organisms. Not only are there surprises in terms of structures and complexity, but interactions ofpotential biological relevance between N-glycans and pentraxins orantibodies could be defined.

(212) Synergistic action of galectin-3 and human α-L-fucosidase 2 in the bactericidal effects of Helicobacter pylori infection

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Helicobacter pylori has persistently colonized over 50% of the global human population. Although most of infected remain asymptomatic yet a minor percentage of people develop gastritis, gastric ulcers, and gastric carcinomas. This is due to the highly stringent host immunity against pathogens and existence of dynamic host-pathogen interplay. More recent studies have shown that galectin-3 is up-regulated and secreted out by the gastric epithelial cells into the surface mucus layer as a response to *H. pylori* infection. This lectin can directly bind to the lipopolysaccharides (LPS), leading to bacterial aggregation and subsequent bactericidal effects. However, fucose decorated O-antigen of *H. pylori* LPS is not a preferred epitope for galectin-3 binding. Thus, it is of significant scientific interest to decipher how fucosylated LPS influences binding affinity of galectin-3 and its effect on galectin-3 mediated bactericidal effects.

In order to evaluate the interaction between galectin-3 and *H. pylori* LPS, we synthesized various fucosylated Lewis antigens (including Le^Y-Le^X, Le^X-Le^X and α 1,2-fucosyl type 2-tetraose) those are similar to O-antigen representing the differential levels of fucosylation.The results clearly demonstrated the modulatory role of fucose, as fucose residues reduced the binding affinity of galectin-3. Our lab previously reported that human FUCA2 is secreted upon *H. pylori* infection. Interestingly, our current data shows that FUCA2 could remove fucose on LPS with the order of cleavage preference as α 1-2 > α 1-3 > α 1-4. Since *H. pylori* LPS has many α 1-3 fucose residues, the preference of cleavage is from non-reducing end of O-antigen. On the other hand, FUCA1, a human lysosomal fucosidase could only remove α 1-2 linkage. Hence, the FUCA2 treatment was able to remove fucose residues on its LPS to expose the poly- or oligo-LacNAc structure that consistently, appeared to be a favored epitope for galectin-3 binding (10-fold higher binding ability, as compared to the untreated LPS). Consequently, the galectin-3-mediated bactericidal effect is increased in FUCA2-treated *H. pylori*. Taken together, these results clearly depict that galectin-3 and human FUCA2 act synergistically to enhance bactericidal effects.

(213) Large Scale N-glycan Preparation from Soy Proteins by Oxidative Release of Natural Glycans (ORNG)

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With escalating interest in glycans and their functions, functional glycomics, which is the systematic study of the glycome, has witnessed significant development in the past decades. However, unlike DNA, RNA or proteins, biosynthesis of glycans are post-translational and not template-driven. Natural glycans are often

complex branched structures that are difficult to access in large scales (milligrams to grams) through either chemoenzymatic synthesis or natural product isolation. We have recently developed a novel approach termed oxidative release of natural glycans (ORNG), in which household bleach can be used to directly release glycans from a large amount (kilograms) of natural materials such as animal/plant tissues. Here we report a large-scale preparation of N-glycans from a kilogram of commercial soy proteins using ORNG. Soy proteins were treated with sodium hypochlorite and the released N-glycans were separated from low molecular weight oxidative product and salts using size exclusion chromatography (SEC). The N-glycan fraction was labeled with the florescent tag, (spell out the name) (AEAB), by reductive amination and separated from excess labeling reagents using size exclusion chromatography. The mixture of glycan-AEAB conjugates was then applied to a preparative amino column, which separated mixture into eight fractions. Each of the eight fractions was separated on preparative scale C18 reverse phase HPLC system for second dimensional purification. From the mixture of released N-glycans we purified 17 N-glycans to apparent homogeneity (>95%), which was confirmed by re-profiling on both C18 and amino columns and subsequent analysis by MALDI-TOF-MS and MS/MS. The major component from this process was 125 mg of $Man_8GlcNAc_2$ with the minor components $Man_{6-7}GlcNAc_2$ and $Man_3GlcNAc_2XylFuc$ ranging from 17 to 59 mg. Six of the purified glycans were characterized by ^1H and/or ^{13}C NMR spectroscopy. Like any degradative process, the ORNG process can generate byproducts from side reactions, which we are working to control to enhance the efficiency of this simple process. To that end, several byproducts were identified, and from these data, we were able to propose potential mechanisms for the oxidative release of natural N-glycans from glycoproteins. Microarray binding assay results confirmed the proposed structures of the purified ORNG released N-glycans. Since the capacity of this process is limited only by the size of the reaction vessels available, the ORNG approach represents a valid, complementary route to synthetic approaches for the preparation of multi-milligram quantities of biomedically relevant complex glycans.

(214) Anthranilic Acid as a Versatile Fluorescent Tag and Linker for Gycan Bioconjugation and Microarrary Preparation

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The advancement of glycoscience is critically dependent on the access to a large number of glycans for their functional study. Naturally occurring glycans is considered a viable source for diverse and biologically relevant glycan libraries. A mixture of free reducing glycans released from natural sources can be fluorescently tagged and separated by chromatography to produce a natural glycan library. Anthranilic acid (AA) has been widely used to fluorescently tag reducing glycans for HPLC or LC/MS analysis. However, AA conjugated glycans are not efficiently immobilized on microarray slides due to the lack of primary alkylamine functional group. In this proposal, we have developed efficient chemistry for bioconjugation and further functionalization of glycan-AA conjugates so that neoglycoproteins and glycan microarrays can be quickly prepared from glycan-AA conjugates, which can be separated by weak anion exchange (WAX) and C18 reverse phase HPLC.

(215) Microbial Exposure Influences the Generation of Anti-Blood Group Antibodies

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Anti-blood group antibodies represent one of the most significant barriers to transfusion and transplantation. However, despite the discovery of ABO(H) blood group antigens and corresponding anti-ABO(H) antibodies over a century ago, the mechanism through which these antibodies form remains relatively unknown. This lack of understanding largely reflects a lack of suitable models capable of defining key players that impact anti-ABO(H) antibody formation. To overcome this limitation, we generated a mouse model that lacks the mouse blood group B disaccharide (B^m). Using this model, we demonstrated that B^mnegative recipients spontaneously develop varying levels of anti-B^mantibodies within the first few weeks of life, similar to humans. To determine whether environmental exposure may impact anti-B^mantibody formation, we specifically housed B^mnegative recipients in sterile conditions to determine whether microbial exposure specifically impacts anti-B^mantibody induction. Remarkably, B^mnegative recipients housed in sterile conditions failed to form any detectable anti-B^mantibodies for over a year, strongly suggesting that microbial exposure may actually be a requirement for naturally occurring anti-B^m antibody development. Consistent with this, cross fostering from sterile conditions to conventionally housed dams allowed normal anti-B^m antibody formation in fostered pups, while non-fostered littermate controls remained anti-B^m antibody negative. Moreover, exposure of adult B^m negative recipients housed in sterile conditions to blood group positive microbes isolated from conventionally housed B^m negative recipients induced anti-B^m antibody sufficient to readily clear B^m positive red blood cells. In contrast, exposure to blood group negative microbes failed to produce any detectable anti-B^m antibody. Taken together, these results demonstrate a vital role for microbial

exposure on the development of anti-B^m antibody in blood group negative mice and provide valuable insight into the mechanism of anti-blood group antibody formation in blood group negative individuals.

(216) Development of a novel yeast cell-based system for studying Core 1-ß3Galactosyltransferase and its Molecular Chaperone Cosmc

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O-glycosylation is one of most common protein posttranslational modifications. O-glycans on glycoproteins play pivotal roles in many biological processes, 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 may contribute to the development and progression of cancer. O-glycans are synthesized by sequential actions of glycosyltransferases. Among them, Core 1 ß3Galactosyltransferase (core 1 ß3GalT, T-synthase) is the key enzyme in the O-glycosylation pathway responsible for converting the Tn antigen to core-1 structure, the precursor of most O-glycans on cell surface. Interestingly, biosynthesis of active Core 1 ß3GalT requires specific molecular chaperone Cosmc (Core 1 ß3GalT Specific Molecular Chaperone), which mainly localizes in endoplasmic reticulum (ER), binds T-synthase, assists its folding andpreventsits aggregation/proteasomal degradation. Alterations in *Cosmc* are associated with human diseases, such as Tn-syndrome, and cancers. However, a detailed understanding of how they affect the molecular interactions between Core 1 ß3GalT and Cosmc remains elusive. To address this matter, we decided to use buddingyeastSaccharomyces cerevisiae, one of the most powerful modelorganisms for studying fundamental aspects of eukaryotic cell biology. We constructed plasmids for expression of T-synthase and Cosmc under control of copper inducible promoter and transformed them into the yeast S. cerevisiae. 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 ß3GalT activity in yeast cells crude extract and demonstrated that similar to mammalian cells T-synthase activity requires presence of its chaperone Cosmc. Protein localization in the yeast cell was investigated through expression of T-synthase and Cosmc fused with Green and mCherry Fluorescent Protein respectively. Proteins were imaged in living cells using fluorescent microscopy. We found that same as in mammalian cells T-synthase and Cosmc colocalize in ER in yeast. 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 indicate that Core 1 ß3GalT protein forms aggregates with amyloid–like features. The details on how Cosmc interacts with Core 1 ß3GalT and possible other proteins/chaperones are under investigation. Our model system will aid to uncover the molecular mechanism behind the human diseases associated with dysfunction of Cosmc.

(217) Pro-survival EGFR signaling and cell surface proteomic analysis of iPS-derived neural crest cells in a human ganglioside deficiency

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Salt & Pepper Syndrome (S&PS) is an autosomal recessive neurological disorder characterized by seizures, severe intellectual disability, choreoathetosis, dysmorphic facial features, and altered skin pigmentation. S&PS is caused by a mutation in the ST3GAL5 gene that encodes GM3 synthase, a glycosphingolipid-specific sialyltransferase. ST3GAL5 synthesizes GM3 ganglioside by adding sialic acid to lactosylceramide. Both GM3 and ST3GAL5 are highly enriched in neural tissues. S&PS and normal fibroblasts were reverted to pluripotency and the resulting induced pluripotent stem cells (iPS cells) were subsequently differentiated to neural crest cells (NC cells) to investigate neural specific glycosylation and signaling alterations associated with loss of GM3. As expected, GM3 is undetectable in S&PS iPS and NC cells. N-linked glycosylation was altered in S&PS NC cells such that the abundance of high-mannose glycans was increased, a phenomenon previously described in cells undergoing apoptosis. Consistent with this glycomic change, increased cleaved caspase 3 was detected as S&PS iPS cells differentiate toward NC. Since glycosphingolipid abundance and glycoprotein glycosylation both modulate receptor signaling in numerous contexts, we assessed the activity of a spectrum of receptor tyrosine kinase pathways. Altered expression and phosphorylation of the Erb family of receptor tyrosine kinases was detected by kinase array and was subsequently validated by western blot analysis. A significant increase in EGFR protein expression and phosphorylation was also detected at the mid-point in NC differentiation, concurrent with the increase in cleaved caspase activity. S&PS iPS cells that escape apoptosis are able to differentiate into NC cells based on the expression of NC markers. However, end-point proteomic analysis of NC cell-surface glycoproteins by Selective Exo-Enzymatic Labeling (SEEL) revealed changes in N-linked glycosylated protein expression in S&PS NC cells compared to normal cells. S&PS iPS cells provide a novel platform to investigate the influence of

glycosphingolipids on cell signaling and cell surface protein presentation in derived cell types.

(218) A potential link between Drosophila Pngl and AMPK signaling

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Mutations in human *N*-glycanase 1 (*NGLY1*) cause a rare developmental disorder with global developmental delay and a host of other phenotypes including neuropathy, movement disorder, and chronic constipation. NGLY1 is a cytoplasmic deglycosylation enzyme capable of removing *N*-linked glycans from *N*-glycoproteins. To understand the pathophysiology of *NGLY1* deficiency, we have characterized mutations in the *Drosophila NGLY1* homolog, *PNGase-like* (*Pngl*). We previously reported that loss of *Pngl* impairs *Drosophila* BMP signaling during intestinal development (Galeone, Han, et al., *eLife*, 2017). However, loss of BMP signaling can only explain some of the intestinal phenotypes in *Pngl* mutants and is not the primary cause of lethality in these animals. One of the *Pngl* mutant phenotypes that cannot be fully explained by the loss of BMP signaling is that they fail to empty their guts at the end of the larval stage. The resulting food accumulation phenotype is likely to contribute to the developmental delay and lethality of *Pngl* mutant larvae. The activity of the visceral muscle (VM) surrounding the midgut is critical for gut clearance. We did not observe gross morphological defects in the *Pngl* mutant midgut VM. However, *Pngl* mutants showed a significant decrease in intestinal peristalsis. It has previously been reported that mutations in *Drosophila AMPKα* result in a food accumulation phenotype in the larva stage associated with decreased gut peristalsis. Accordingly, we examined whether decreased AMPK signaling can explain the food accumulation and lethality in *Pngl* mutants. I will present genetic and biochemical evidence suggesting a potential link between Pngl and the energy sensor AMPK.

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(219) Identification of glycoprotein and glycan cell surface markers for ventricular-, atrial- and nodal-like cardiomyocytes

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Heart failure and coronary heart diseases are a major cause of death, especially in western industrialized countries (Townsend et al., *Eur. Heart J.*, 2015). In case of terminal heart failure, heart transplantation is the only possible cure, but donor organs are scarce. Therefore, recent approaches in regenerative medicine aim at replacing damaged cardiac tissue by pluripotent stem cell-derived cardiomyocytes. Importantly, the heart comprises several subtypes of cardiomyocytes, i.e. ventricular, atrial and nodal (pacemaker) cardiomyocytes, differing in their spatial distribution, gene expression and physiological properties. Thus, depending on the patient's disease, grafts with specific subtype compositions will be needed. However, recent differentiation protocols do still not produce pure populations of specific cardiomyocyte subtypes from pluripotent stem cells. Moreover, due to a lack of cell surface markers, further purification e.g. by cell sorting is not possible yet. Therefore, our aim is to identify glycoproteins or glycans located specifically at the cell surface of ventricular-, atrial- or nodal-like cardiomyocytes, which can be used as markers for the different cell types. To achieve this, we apply a CRISPR/Cas9-mediated knock-in of GFP in human induced pluripotent stem cells (hiPSCs) downstream of genes known to be specifically expressed in the different cardiomyocyte subtypes. Upon cardiomyogenic differentiation of the modified hiPSCs, specific cardiomyocyte subtypes will be enriched based on GFP expression and will be used to study the glycoproteome and glycome in order to identify novel cell surface markers for ventricular, atrial and pacemaker cardiomyocytes.

(220) Defining the impact of O-GlcNAc on fertility of C. elegans males

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NIH / NIDDK

Fertility is impacted by environmental factors including diet, xenobiotics, and stress. O-GlcNAc is an important post-translational modification involved in diverse pathways including nutrient signaling, stress response, and sperm biology. Though lethal in most metazoans, loss of function of O-GlcNAc transferase (*ogt-1*) is viable in *C. elegans*, though these animals are devoid of O-GlcNAc-modified proteins. Though hermaphrodite *ogt-1* mutants appear generally healthy and their fertility is not significantly compromised, *ogt-1* males have a four-fold reduction in mating success. This is striking since spermatogenesis occurs in both sexes, though there are several well-characterized differences. Males produce larger sperm that outcompete hermaphrodite sperm and have unique signaling pathways that regulate spermatogenesis and mating. Despite what is known about *C. elegans* male biology, the mechanistic link between O-GlcNAc and fertility is unclear.

Development of the male copulatory organ may play a role in the *ogt-1* phenotype, as our analysis found a significant increase in developmental defects at a low penetrance. Sperm tracking assays demonstrated that a reduced number of sperm from *ogt-1* males enters hermaphrodites, which may result from aberrant sperm motility or defective mating behavior. These data suggest developmental and behavioral defects are contributing to reduced fertility, but another process is likely to be a key factor.

Sperm count and morphologic defects in sperm activation will be determined. Motility will be assayed with live imaging both *in vitro* and *in vivo* by migration to the hermaphrodite spermatheca. We will exploit the genetic amenability of *C. elegans* to test the contributions of different candidate tissues to the fertility defect. We will test the tissue-specificity of the *ogt-1* phenotype with tissue-specific rescue experiments. We have found that *ogt-1* males often fail to transfer sperm and have an increase in tail defects, which suggest the possibility of a problem in mating behavior. To test this, we will conduct assays of the well-characterized, stereotypical behaviors of male mating. Further experiments using genetic and biochemical tools will be used to dissect experimental findings.

(221) Improved and semi-automated reductive β-elimination workflow for higher throughput protein O-glycosylation analysis

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Protein *O*-glycosylation has shown to be critical for a wide range of biological processes, resulting in an increased interest in studying its alterations in biological samples as disease biomarkers as well as for patient stratification and personalized medicine.

Whilst *O*-glycosylation has been reported to be a common post-translational modification of proteins, it is demanding from an analytical point of view. *O*-glycan changes can be complex and subtle, often requiring a large number of samples to be analysed in order to obtain conclusive results.

Although considerable progress has been made with respect to rapid sample preparation, method integration and robotization decreasing the hands-on time required to prepare and measure glycan samples, there is still a considerable gap between *O*- and *N*-glycan glycomics in terms of throughput. Most of the *O*-glycan analysis work done so far has been performed using glycoanalytical technologies that would not be suitable for the analysis of large sample sets, mainly due to limitations in sample throughput, resolution and affordability of the methods. The development of automated workflows is necessary to reduce the time required to prepare and measure *O*-glycan samples as well as provide a system that is easy to operate, repeatable and reliable.

Automated workflows can be obtained by using liquid handling robots for sample processing, cleanup and sample preparation. Automated platforms can take over many of the time-consuming and labour-intensive steps, providing similar performance to expert manual labour and even outperforming manual sample preparation when large cohorts are measured.

Here we report a largely automated system for *O*-glycan analysis. We adapted the reductive β-elimination release of *O*-glycans to a 96-well plate format and transferred the protocol onto a liquid handling robot. Release of *O*-glycans for 96 samples was achieved within 2.5 hours and post-release purification and derivatization by permethylation was performed in 10 hours. The automated data acquisition on MALDI-TOF-MS took less than 1 minute per sample. The method has been validated according to the ICH Q2 (R1) guidelines using a BSM (bovine submaxillary mucin) type I-S glycoprotein standard that contains complex and heterogeneous types of *O*-glycans. Results of the automated method were in good agreement with the manual reference system and even outperformed it in terms of repeatability, being able to produce lower CVs.

To our knowledge, this is the first largely automated workflow for *O*-glycan release that has been adapted for its execution on a liquid handling robot.

The method has been successfully applied to characterize *O*-glycans of colorectal cancer cell lines and biopharmaceutical products. The method has high-throughput potential and is suitable for the characterization and relative quantitation of *O*-glycans from biological samples as well as biopharmaceuticals.

(222) Development of Anti-Glycan VLRB Libraries from Immunized Lampreys

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Studies on the expression and structure of cellular glycans are limited by the lack of sensitive tools that can discriminate specific structural features. Here we describe the development of a robust platform using immunized lampreys (*Petromyzon marinus*) for generating libraries of anti-glycan variable lymphocyte receptor B (VLRB). Adjuvant-free immunization with whole fixed cells, tissue homogenates, and human milk generated a wide variety of target-specific antibodies detectable in lamprey plasma. The cDNAs from lamprey lymphocytes were cloned into yeast surface display libraries for enrichment, and monoclonal VLRB-Ig chimeras were produced for screening and characterization. By this approach, we generated a dozen monoclonal VLRBs that recognize specific glycan antigen epitopes as profiled by glycan microarrays. These reagents, termed smart anti-glycan reagents (SAGRs), are useful for all types of glycan analyses, including Western blots and flow cytometry. Comparative amino acid sequences of each VLRB suggest specific sequences determine specificity for interactions. The use of SAGRs will help to elevate and enhance future studies on glycan expression by providing sequenced antibodies for use in many types of research formats.

(223) O-GlcNAc regulates intestinal stem cell proliferation and gut regeneration in Drosophila

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The O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) regulate the dynamic cycling of the post-translational modification O-GlcNAc in a nutrient- and stress-responsive manner. O-GlcNAc contributes to tissue development and is implicated in cancer malignancy. *Drosophila* is a powerful model system for studying adult stem cell biology and findings have implications for aging-related diseases and stem cell-derived diseases, including cancers. *Drosophila* intestine is high conserved with mammal intestine with respect to the signaling pathways that control intestinal development, regeneration and disease. *Drosophila* intestine stem cells (ISCs) are critical for tissue maintenance and homeostasis under nutrient- and stress-responsive conditions, such as oxidative stress. However, the role of O-GlcNAc in intestinal stem cells remains unknown. Our findings indicate that O-GlcNAc levels increase in intestinal stem cells under aging and oxidative stress-related conditions. In addition, increased O-GlcNAc (by knockout of OGA) leads to O-GlcNAc expression, ISC hyperproliferation, activation of DNA damage repair, and AKT/TOR expression in the *Drosophila* midgut. Deficiency of O-GlcNAc (by knockout of OGT) did not increase ISC proliferation and DNA damage repair activity in intestinal stem cells under oxidative-related stress condition. These studies identify that O-GlcNAc is important for intestinal stem cell proliferation and gut regeneration. Therefore, our studies underscore the read to define the role of O-GlcNAc plays in age-related diseases and adult stem cell-derived diseases, such as cancer.

(224) Paired Data Analysis of Glyco-gene Transcripts and Glycan Structural Data Derived from Differentiated Human Stem Cell Lineages

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The differentiation of human embryonic stem cells (hESCs) is being used a model to study the changes in glycan-related gene transcripts and glycan structural changes that occur during cellular differentiation. A combination of high-throughput qRT-PCR and RNA-Seq data were collected for ~900 glycan-related genes 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. Previously, we developed a pathway visualization tool to address this issue through the use of the Metscape plug-in associated with the open source software Cytoscape for analyzing trimming and branching of high mannose*N*-linked glycosylation. 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 this unified platform for the display of both data types allows the identification of correlative changes that occur during cellular differentiation. The pathway visualization framework has now been extended to include capping reactions such as sialylation, fucosylation and poly-lactosamine extension of the core*N*-glycan structures to compare results from glycan structural analysis and transcript abundance data 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). These differentiated cell lineages encompass 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).

(225) The Development and Characterization of Antibodies to Site-specific O-GlcNAc modified Histones for Epigenetic Research

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O-glycosylation of nuclear and cytoplasmic proteins by a single β-N-acetyl-D-glucosamine moiety (O-GlcNAc) is a common post-translational modification that is highly dynamic and fluctuates in response to cellular stimuli. This type of glycosylation has been found on approximately a thousand human proteins to date and is thought to be nearly as widespread and abundant as protein phosphorylation. Many of the first proteins identified carrying this modification were transcription factors, and it became apparent in the last several years that O-GlcNAc plays a significant role in chromatin remodeling and gene expression.

Given the complexity of the histone code, site-specific antibodies are needed to begin to elucidate the hierarchy of modifications as well as the biological significance of single and multiple post-translational modifications of the histones. We have developed and approach to provide high-affinity site-specific O-GlcNAc antibodies that appear to overcome issues related to small carbohydrates being poor antigens and the O-GlcNAc modified epitopes being self-antigens. These site-specific antibodies require a more elaborate characterization scheme than other antibodies as they need to demonstrate both a preference for the O-GlcNAcylated species over the native peptide sequence and a specificity to the amino acid sequence. We use a multi-step evaluation process to characterize our antibodies. 1) ELISA assay, to determine the preference of the antibody to the O-GlcNAcylated peptide over the unmodified form; 2) Cross-reactivity of the antibody to other peptide sequences modified with O-GlcNAc; 3) Immunoprecipitation increasing wash stringencies and 4) Evaluation of the site-specific O-GlcNAc histone antibodies for their potential as ChIP-Seq reagents. Using this process, we have authenticated our site-specific O-GlcNAc antibodies and have demonstrated that they possess the preference and specificity needed for epigenetic research. The work was supported by R44GM110887.

(226) A Molecular switch orchestrates enzyme specificity and secretory granule morphology

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NIDCR/National Institutes of Health

Regulated secretion is an essential process where molecules destined for export are directed to membranous granules, where they undergo packaging and maturation. However, many of the factors that influence cargo packaging and granule maturation remain ill-defined. Here, we identify a novel gene (*pgant9*) that influences the structure and shape of secretory granules within the *Drosophila* salivary gland. Loss of *pgant9*, which encodes an O-glycosyltransferase, resulted in secretory granules that had an irregular, shard-like morphology and altered glycosylation of granule cargo. Interestingly, *pgant9* undergoes splicing to generate 2 isoforms with distinct substrate specificities. Structural determination by X-ray crystallography reveals that this splicing event acts as a molecular switch that alters the charge of a loop controlling access to the active site of the enzyme. The splice variant with the negatively charged loop rescues secretory granule morphology and is able to glycosylate the positively charged secretory cargo of the salivary gland. Our study has uncovered a novel mechanism for dictating substrate specificity within the O-glycosyltransferase enzyme family. Moreover, our in vitro and in vivo studies suggest that the glycosylation status of secretory cargo influences the morphology of maturing secretory granules. Finally, the molecular switch between positively and negatively charged loops may have evolved to ensure appropriate glycosylation of charged cargo present in various tissues.

(227) Altered starch in corn results in masa not suitable for tamales

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Tamales made from masa prepared by a well known market in Downey, CA did not hold together and fell apart. This was a large batch sold to several hundred customers. As a result, the store suffered serious negative publicity which impacted their business. The corn used in the preparation of the masa was suspect. Masa is prepared by first nixtamalization of corn which involves cooking for a short period in a solution of $CaOH_2$ followed by several hours of steeping which loosens the pericarp. Next the $CaOH_2$ is washed out andthe corn is ground and mixed with other ingredients to become masa. The masa then becomes the outer layer of the tamale inside the corn husk wrapping. The masa appeared to be normal but on the cooking of the tamales the problem appeared. During the second cooking the starch goes through a gelatinization in which starch granules take up water, swell and amylose and other glucans are leached out. The gel with the glucans are components contribute to the binding. Due to widespreadnon–GMO publicity there was suspicion of contamination by a GMO corn, Enogen, which has an active heat stable amylase to facilitate production of ethanol. Samples of known good corn and the suspect corn were compared. Assays involving incubations at 37, 55 and 80° for 18, 36, and 68 hoursdid not reveal any amylase activity. Starchy endosperm was excised from the nixtamalized corn, homogenized and cooked for 30 min increments with the supernatant changed each time. There is a very significant deficiency of glucan polymers released from the starch of the bad corn compared to the good corn. The deficiency is in the aqueous extract as well as the aqueous component of the gel fraction which is involved in the binding in the tamales. The suspected cause of the problem is unusually high temperatures in the field which inhibits the enzymes involved in starch synthesis at a critical time late in the season. This case was the subject of major legal action. Subsequent investigation has revealed that this was not an isolated case but other cases were not investigated and assumed to be due to Enogen contamination. With global warming this may become more common.

(228) The Identification and Analysis of Two Arabidopsis Proteins Responsible for the Methylation of Glucuronosyl Side Chains of Arabinogalactan Polymers

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Arabinogalactan Proteins(AGP) are plant cell wall resident glycoproteins that contain polysaccharide polymers linked to hydroxyproline residues of a small protein backbone. In *Arabidopsis*, these AG polysaccharides are typically composed of a backbone structure of β1-3 linked galactans that are substituted regularly with β1-6 linked galactan sidechains. These side chains are often further decorated with α and β arabinosyl residues as well as other carbohydrates such as fucose, rhamnose and glucuronic acid, the latter of which is often found methylated at the 4' hydroxyl. Recent studies have demonstrated that methylation of the terminal glucuronic acid residues of AGP's is critical in signaling events during plant reproduction and growth. Here we show that two *Arabidopsis* genes are essential for the addition of methyl substituents to AGP GlcA residues *in vivo*. Expression analysis of these two homologous genes, named agmt1 and agmt2, demonstrate that while one is expressed ubiquitously throughout all examined tissue types, the other is specifically expressed within the roots of liquid cultured *Arabidopsis* plants. Extracted AGPs from *Arabidopsis agmt1xagmt2* null mutants were analyzed via H^1 NMR and GC-MS and were found to lack 4-O methylation of glucuronosyl side chains when compared to wild type plants. Furthermore, tissue specific reductions in GlcA methylation were analyzed in *agmt1xagmt2* single and double mutants and showed that nullification of a single homologue can result in tissue specific affects. Analysis of extracted xylans and xyloglucans from the *agmt1xagmt2* double mutant displayed no differences in methylation content or structure, suggesting that AGMT1 and AGMT1 act specifically on arabinogalactan polymers. Together, this data suggests that AGMT1 and AGMT2 most likely function as methyl transferases acting on glucuronosyl residues attached to arabinogalactan polymers. (This research was supportedby The Center for Bioenergy Innovation (CBI),a U.S. Department of Energy Research Center supported by the Office of Biological and Environmental Research.We alsothank the Center for Plant and Microbial Complex Carbohydrates (DESC0015662) for equipment support.)

(229) Molecular recognition and catalysis of a health-promoting beta-glucan by prominent human gut Bacteroidetes

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Gastrointestinal health is a cornerstone of our wellbeing and the composition and physiology of the gut microbiota is heavily influenced by the influx of complex polysaccharides (commonly known as "dietary fibre") from our diet. Mixed-linkage β-glucan (MLG) from cereals, in particular, has been linked to a wide range of health benefits, including reduction of blood cholesterol levels and mitigating metabolic syndrome [1]. Members of the Bacteroidetes, a dominant phylum in the human gut, possess an arsenal of polysaccharide utilization loci (PUL) to target a wide range of complex polysaccharides. We have identified a PUL in *Bacteroidesovatus* that is transcriptionally upregulated in the presence of, and necessary for growth on, barley MLG. Through combined biochemical, enzymatic, biophysical and structural characterization, we have illuminated key aspects of the molecular mechanism by which *B. ovatus* utilizes β-glucan [2].

Our collection of crystal structures of the two outer membrane glycan-binding proteins reveal the molecular basis underlying MLG specificity in conjunction with biophysical studies. We similarly reveal structural basis for specific and efficient MLGase activity of a cell surface-localized glycoside hydrolase (GH) family 16 enzyme. Small oligosaccharides that are liberated from the large MLG polysaccharide at the cell surface are then imported to the periplasm by a TonB-dependent transporter. There, the specific breakdown products activate transcription of the PUL via the hybrid two-component sensor [3] and are eventually reduced to monosaccharides by the action of a GH family 3 exo-glucosidase. Together, these data allow us to outline a pathway by which these key PUL gene products act in concert to efficiently capture and metabolize dietary MLG in the competitive distal gut environment. On a population level, we further reveal through (meta)genomics that the relevance of MLG metabolism by gut symbionts extends to the majority of humans regardless of nationality or disease state.

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