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PROGRAM AND ABSTRACTS FOR 2016 ANNUAL MEETING OF THE SOCIETY FOR GLYCOBIOLOGY

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SFG 2016 POSTER PROGRAM

Session 1: Using model systems to understand the biological roles of glycans

Session 3: Glycan foraging by vertebrates and microbes

Session 4: Host-pathogen interactions

Poster Number *Abstract Number*

Session 5: Prokaryote versus eukaryote glycobiology: similarities and differences

Poster Number *Abstract Number*

Session 6: Glycans in development and genetic disorders

Poster Number *Abstract Number*

Session 7: Relevance of carbohydrates in disease, diagnosis, prevention and treatment

Session 8: New tools and their applications

Poster Number *Abstract Number*

Session 9: Glycans and glycan binding proteins in immunity

Poster Number *Abstract Number*

(1) Control of vesicle trafficking by intracellular glycosylation: From chemical biology to vertebrate development

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One third of all eukaryotic proteins pass through the secretory pathway for targeting to specific locations, including the endoplasmic reticulum (ER), Golgi, plasma membrane or extracellular milieu. Since misdirected proteins cannot function, the secretory pathway plays a critical role in establishing and maintaining normal cell and tissue physiology. In particular, the COPII coat protein complex, which mediates vesicle trafficking between the ER and Golgi, is a key control point for protein targeting. Moreover, mutations in COPII genes cause a range of human diseases, including craniofacial defects and hematological disorders. Detailed knowledge of COPII vesicle trafficking is required to understand its role in cell physiology and to devise new treatments for disorders in which it is disrupted. However, while the core COPII machinery is well defined, little is known about how mammalian cells regulate COPII activity in response to developmental, metabolic or pathological cues.

Recently, we and others found that several COPII proteins are modified by O-linked β-*N*-acetylglucosamine (O-GlcNAc), a dynamic form of intracellular glycosylation. However, the mechanistic and functional impacts of O-GlcNAc on the COPII pathway remain unclear. We used a chemical biology approach to show that at least four COPII components engage in O-GlcNAc-mediated protein-protein interactions in human cells, and that pharmacological inhibition of O-GlcNAc cycling impairs COPII trafficking. These results indicate that O-GlcNAc regulates COPII activity through the modification of specific pathway components. We focused on Sec23 because it is essential for COPII trafficking and because Sec23A mutations cause cranio-lenticulo-sutural dysplasia (CLSD) in humans. Mass spectrometry and mutagenesis identified eight sites of O-GlcNAcylation on Sec23A, including four required for the above-mentioned O-GlcNAc-mediated protein-protein interactions. Interestingly, a subset of unglycosylatable Sec23A mutants failed to rescue a Sec23A loss-of-function zebrafish mutant, recapitulating CLSD-like craniofacial defects. Our results suggest that O-GlcNAc dynamically regulates COPII through Sec23A and perhaps other substrates, and may reveal new opportunities for future therapeutic intervention in human diseases of protein trafficking.

(2) Self-glycans in autoimmune disease

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The innate immune system is the first line of defense against invading pathogens. One important feature of innate immune recognition is self versus non-self discrimination. Erroneous recognition of self-ligand often leads to constitutive activation of innate immune signaling resulting in autoimmune disease. We study TREX1, which is an endoplasmic reticulum (ER)-associated exonuclease and a critical negative regulator of innate immunity. TREX1 mutations are associated with several autoimmune and autoinflammatory diseases, including Aicardi–Goutières syndrome (AGS), familial chilblain lupus (FCL), systemic lupus erythematosus (SLE), and retinal vasculopathy with cerebral leukodystrophy (RVCL). Biallelic mutations abrogating DNase activity cause autoimmunity by allowing immunogenic self-DNA to activate type I interferon (IFN) response. We recently showed that dominant TREX1 frame-shift (fs) mutations disrupt a DNase-independent function associated with TREX1 C-terminus. We found that TREX1 C-terminus suppressed immune activation by interacting with the ER oligosaccharyltransferase (OST) complex and stabilizing its catalytic integrity. C-terminal truncation of TREX1 by fs mutations dysregulated the OST complex, leading to release of free glycans, immune activation and autoantibody production. We recently established the TREX1-V235fs knock-in mouse to better mimic the human disease. Our ongoing study begins to reveal broader mechanisms by which self-glycans could trigger chronic immune activation and autoimmune disease.

(3) Uniquely Human Genetic Changes in Sialic Acid Biology: Implications for Human Evolution and Disease

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Chimpanzees amino acid sequences are generally >99% identical to humans (more similar than mice and rats). Thus, biological differences between these "hominids" were thought to arise from gene expression differences. In 1998, we reported the first human-unique and human-universal genomic mutation with a biochemical consequence. This event altered sialic acids (Sias) on almost all cell-types, and may have protected against hominid malarias. This inactivating exon deletion in *CMAH* occurred ~3-4 mya, with a coalescence time of ~2-3 mya. The resulting immune reaction against the non-human sialic acid Neu5Gc may have contributed to speciation of *Homo*, by anti-Neu5Gc-antibody-mediated cryptic female choice against sperm and embryos that were Neu5Gc-positive. We also discovered a human-unique pattern of expression of alpha2-6-linked Sia expression on epithelia that may explain human resistance to avian influenza viruses. Such alterations in the human "sialome" may have triggered additional human-specific changes in Sia biology, which affect >10 of the <70 genes involved in synthesis, recognition and turnover of Sias. We found multiple human-specific and human-universal differences in Siglecs (Sialic acid-recognizing immunoglobulin-like

lectins)—including binding specificity changes (in Siglecs-5,-11,-12 and -14); expression pattern changes (in Siglecs-1,-5,-6,-11 and -14); gene conversion (*SIGLEC11*); gene deletion (*SIGLEC13*) and pseudogenization (*SIGLEC17*). Human-unique and human-population-universal polymorphic pseudogenization of *SIGLEC12*, *SIGLEC14* and *SIGLEC16* suggest ongoing selection. Human-specific consequences include Siglec-11 expression in microglia, Siglec-6 expression on the placental trophoblast, suppression of Siglec-5 expression on adaptive immune cells, and elimination of Siglec-13 and -17 from innate immune cells. The latter events seem to have occurred shortly before the origin of humans ~100-200 kya, and resurrected gene products bind potentially lethal pathogens of infants. While such pathogens may have contributed to population bottlenecks, the resulting changes in Sia biology may have also altered multiple systems wherein Sias and Siglecs have endogenous roles. A non-genetic result of the CMAH mutation is human metabolic incorporation of foreign dietary Neu5Gc despite circulating anti-Neu5Gc antibodies, a novel xeno-autoantigen situation. Thus, Sia biology-associated genes comprise a relative hot spot of genetic and physiological changes during human evolution, with implications for human origins and uniquely human features, in health and disease.

(4) Ultra-deep genome mutagenesis in haploid human cells

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Loss-of-function forward genetics are a powerful tool for linking genes to phenotypes in model organisms. Yeast mutants and lectin-selected CHO cells have, for instance, yielded fundamental insights into diverse glycosylation pathways. Unfortunately, the systematic application of loss-of-function genetics to human cells has been hindered by technical barriers for a long time. Advances in programmable bacterial nucleases and ultra-deep genome mutagenesis of haploid human cells can overcome this hurdle.

In our group, we use these tools to study the genetic basis of human disease. Despite decades of biomedical research, the genetic contribution to many disease phenotypes remains uncharted or incompletely understood. Moreover, even if genes can be linked to a particular phenotype, their interactions with other genes are rarely known. This constitutes a bottleneck for therapeutic intervention, as a detailed understanding of the genetic crosstalk underlying a disease phenotype could offer novel treatment avenues.

Our goal is to use loss-of-function genetics in cultured human cells to generate exhaustive and accurate maps of genes involved in phenotypes of interest and to understand their interactions. To this end we have developed a new genetic model system that enables yeast-like genetics inhaploid human cells. We used this, for instance, to identify genes needed for the generation of the laminin-binding O-glycan of dystroglycan and host factors used by pathogens, which includes a new class of intracellular receptors required by Ebola and Lassa virus, alongside an unexpected glycan preference in the latter case. We also combine CRISPR/Cas and ultra-deep mutagenesis of haploid cells to monitor fitness effects of tens of millions of mutant alleles under various phenotypic selection criteria and in different mutant backgrounds. This allows us to study the principles of genetic interactions inhuman cells. In a pilot project, we created a mutation-based synthetic lethality map focusing on the secretory pathway. This revealed similarities of human genetic interactions to yeast in terms of abundance and compartmentalization. It also allowed us to assign functions to previously orphan genes (such as TMEM258, a novel component of the OST complex), and projects a route forward to decipher genetic interactionsaffecting diverse aspects of human cell biology.

(5) TBD

Michael Tiemeyer

Abstract to come

(6) Understanding complex glycan utilization in the human microbiota

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The human large bowel is colonized by a community of microbes, the microbiota, which has a significant impact on human health and nutrition through the production of short chain fatty acids (SCFAs), and by interaction with the host immune system. The major nutrients available to these organisms are dietary glycans, also known as complex carbohydrates. Thus, dietary and nutraceutical strategies, based on complex carbohydrates, can, potentially, be deployed to encourage the dominance of beneficial microbes in the microbiota, particularly those producing health promoting SCFAs such as propionate and butyrate, and bacteria that have an anti-inflammatory impact through its interaction with the human immune system, ensuring that this microbial ecosystem has a positive influence on human health. This

approach, however, is greatly restricted by a critical lack of understanding of the mechanisms by which complex glycans are metabolized by the microbiota. Significantly, the wealth of genomic and metagenomic microbiota sequence presents an exciting, but so far unfulfilled, opportunity to make decisive advances in our understanding of glycan metabolism in the human large bowel. This seminar will review our biochemical, genetic and microbiological strategies, in harness with metagenomic and genomic data, to understand the mechanisms of complex glycans utilization by the human microbiota1,2,3. The models established, will trigger the development of novel dietary strategies that are designed to maximize human health through manipulation of microbiota structure.

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(7) The role of intramolecular trans-sialidases in intestinal mucin-degrading bacteria

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The gastrointestinal tract is inhabited by a diverse microbial community (microbiota) that influences host health. The large intestine is lined by a mucus bi-layer, with the outer layer providing a habitat to bacteria whereas the inner layer keeps them away from the epithelium surface. The terminal mucin O-glycans can serve as attachment sites and metabolic substrates to the gut commensal bacteria which have adapted to the mucosal environment. So far, only a limited number of intestinal bacterial species/strains from the Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia phyla have been studied for their ability to consume mucins but it is becoming apparent that the strategy used by these mucin-degraders is species and strain-dependent.

Ruminococcus gnavus is a prominent member of the gut microbiota, belonging to the Firmicutes phylum. We previously showed that the ability of *R. gnavus* to utilize mucins was strain-dependent and associated with sialic acid metabolism *via* the expression of an intramolecular *trans*-sialidase (*Rg*NanH) that cleaves off terminal α2–3-linked sialic acid from glycoproteins, releasing 2,7-anhydro-Neu5Ac instead of sialic acid. *Rg*NanH is a multi-domain enzyme with a catalytic domain from the glycoside hydrolase family 33 (*Rg*GH33) and a carbohydrate-binding module of family 40 (*Rg*CBM40).

Here we further explored *R. gnavus* mucin-degradation strategy by genomics and transcriptomics analyses and confirmed the ability of the strains to grow on 2,7-anhydro-Neu5Ac. Using a combination of biochemical, structural and cell based studies, we deciphered the substrate specificity of *Rg*NanH and the contribution of *Rg*GH33 and *Rg*CBM40 in targeting sialylated rich intestinal mucins. Together the data suggest that intramolecular *trans*-sialidases may provide gut microbes such as *R. gnavus* with a competitive nutritional advantage, allowing the bacteria to thrive within mucosal environments by scavenging sialic acid from host mucus in a form, 2,7-anhydro-Neu5Ac, that can be used to their own benefit.

(8) Structural basis for glycan acquisition by dominant members of the human gut microbiota

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The gut microbiota plays an important role in human health and nutrition. The survival of microbiota members from the dominant Gram-negative phylum Bacteroidetes depends on their ability to degrade dietary glycans that cannot be metabolised by the host. Glycan degradation occurs mainly intracellularly and depends critically on the import of intermediate-sized oligosaccharides by an outer membrane (OM) protein complex composed of an extracellular SusD-like lipoprotein and an integral membrane SusC-like transporter. Many sequenced gut *Bacteriodes* spp encode over 100 C/D pairs, with the majority of them having unknown functions and substrate specificities. A central, unresolved question is how extracellular substrate binding by SusD proteins is coupled to OM passage via their cognate SusC transporter. Here I will present data on the crystal structures of two functionally distinct SusCD complexes purified from *Bacteroides thetaiotaomicron* and using these data in combination with molecular dynamics simulations and single channel electrophysiology derive a general model for substrate translocation. Our results provide mechanistic insights into OM nutrient import by members of the microbiota, which is of major significance for understanding the human-microbiota symbiosis.

(9) A molecular view of glycan utilization by the human gut microbiota

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Intestinal bacterial forage on a vast array of dietary and host-derived carbohydrates. Our research seeks to understand the molecular basis of carbohydrate recognition and import for a variety of dietary fibers, with a specific focus on starch. The Gram-positive Firmicutes comprise a significant portion of the mammalian gut microbiota and encode large multi-domain glycoside hydrolases which combine glycan

binding and processing on a single polypeptide chain. Liberated oligosaccharides are captured by separate ATP-binding cassette or phosphotransferase systems for import across the cell membrane. Our work comparing the biochemical and structural features of the starch-targeting enzymes of *Ruminococcus bromii* and *Eubacterium rectale* demonstrates how these organisms may be uniquely suited for starch utilization compared to other bacteria. A mechanistic understanding of glycan selection and import by gut bacteria is necessary for the rational design of non-invasive dietary-based strategies for optimizing the gut community to improve health.

(10) Regulation of intestinal dysbiosis by bacterial sialidases

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Imbalance in the gut microbial community, as exemplified by expansion of *Enterobacteriaceae*, is frequently observed during intestinal inflammation. However, the mechanisms underlying such bacterial shifts remain elusive. We have demonstrated that the carbohydrate sialic acid contained in the mucus layer lining the colon is a critical factor contributing to the outgrowth of *Enterobacteriaceae* during intestinal inflammation in mice. The release of sialic acid conferring growth advantage for *Enterobacteriaceae* was regulated by bacterial sialidase enzymes, which activity increased significantly during inflammation. We found that *Bacteroides spp.* were a major source of sialidase activity in the caecum fluid of mice. The expansion of *Enterobacteriaceae* during inflammation further aggravated colitis by stimulating the production of the pro-inflammatory cytokines IL-6, IL-12, and TNF-alpha by intestinal leukocytes. The inhibition of bacterial sialidase activity prevented *Enterobacteriaceae* proliferation and decreased the severity of colitis in treated mice. The beneficial effect of sialidase inhibition on intestinal inflammation suggests new therapeutic approaches targeting intestinal dysbiosis as manifested by *Enterobacteriaceae* expansion.

(11) The glycosylation strategies of giant viruses: old tools for new functions

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Many viruses have glycosylated proteins and the glycan portion of the glycoproteins is known to playfundamental roles in protein folding, progression in the secretory pathway and regulation of host–virus interactions. Eukaryotic viruses studied to date use host-encoded enzymesof the endoplasmic reticulum and Golgi apparatus for glycoprotein synthesis and processing. Thus, the oligosaccharide structurescomposing the glycoproteins and the virions are related to those of host cells. However, several recent reports indicate that large and giant viruses belonging to the Phycodnaviridae and Mimiviridae families encode most, if not all, of the glycosylation machineries, which differ from the previously characterized systems used by Eukaryotes and Prokaryotes. This results in the formation of novel and unusual glycan structures. For glycosylation, three requirements need to be met: (i) availability of nucleotide-sugar precursors; (ii) the presence of glycosyltransferases, glycosidases and other enzymes for glycan synthesis and remodeling; and (iii) a defined area of the cell where synthesis can occur. Enzymes for nucleotide-sugars synthesis have been characterized in Chloroviruses and Mimiviruses. Several glycosyltransferases and other related enzymes have been identified in the genome of these viruses and the structural characterization of some virus-associated glycans is now providing the basis to analyze their function. Genome inspection of the several newly identified giant viruses reveals that the presence of glycoenzymes is not restricted to a few species, but widespread in giant viruses from all environments and families, suggesting an important role of glycans for virus stability and interaction with their eukaryotic hosts.

However, the question of where and how protein glycosylation occurs during giant virus assembly is a major challenge for future research. Another question is to identify all of the viral genes involved in the glycosylation process and to determine the evolutionary origin of these genes; this latter issue falls in the more general problem regarding the role of viruses in gene transfer between prokaryotes and eukaryotes and in protein evolution. Indeed, the studies on giant virus glycosylation can also provide clues on how an independent glycosylation system can develop and evolve.

(12) Outer Membrane Vesicles of friends and foes

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Outer membrane vesicles (OMV) are spherical membranous structures released from the outer membrane (OM) of Gram-negative bacteria. OMV have been proposed to play several different roles during both pathogenesis and symbiosis. Whether OMV are produced by an active mechanism or by passive disintegration of the OM is a still matter of controversy. We first addressed this question in *Bacteroides fragilis* and *B. thetaiotaomicron*, two important members of the human microbiota. Proteomic analysis of OM and OMV

in B. fragilis identified more than 40 proteins found exclusively in OMV and more than 30 proteins detectable only in the OM. The OMV-specific proteome showed a high prevalence of glycosidases and proteases, some of which were shown to be active in vitro. Similar results were obtained for B. thetaiotaomicron. Most of the OMV-exclusive proteins were acidic. Based on these results, we propose that these species possess machinery devoted to selectively pack acidic proteins into the OMV. These OMV equipped with hydrolytic enzymes could help in securing nutrients for the benefit of the whole bacterial community present in the microbiota, uncovering a novel function for bacterial OMV. Despite the fact that OMV were described several decades ago, their biogenesis is a poorly characterized process. It has been shown in eukaryotic membranes that lipids with an inverted-cone shape favor the formation of positive membrane curvatures. Based on these studies, we formulated the hypothesis that lipid A deacylation might impose shape modifications that result in the curvature of the outer membrane (OM) and subsequent OMV formation. We tested the effect of lipid A remodeling on OMV biogenesis employing Salmonella enterica serovar Typhimurium as a model organism. Expression of the lipid A deacylase PagL resulted in increased vesiculation, without inducing an envelope stress response. Mass spectrometry analysis revealed profound differences in the patterns of lipid A in OM and OMV, with accumulation of deacylated lipid A forms exclusively in OMV. OMV biogenesis by intracellular bacteria upon macrophage infection was drastically reduced in a pagL mutant strain. We propose a novel mechanism for OMV biogenesis requiring lipopolysaccharide (LPS) remodelling in the context of a multifactorial process that involves the orchestrated remodeling of the outer membrane.

(13) TBD

Jorge Galan

Abstract to come

(14) N-linked protein glycosylation in pro- and eukaryotes

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N-linked protein glycosylation is a major modification of secretory proteins that can be found in all three domains of life; archaea, bacteria and eukaryotes. In these homologous pathways, the assembly of an oligosaccharide takes place at the cytoplasmic side of the prokaryotic plasmamembrane or the membrane of the Endoplasmic Reticulum, respectively. Isoprenoid lipids serve as membrane anchors and, after translocation across the membrane and further extension of the oligosaccharide in a majority of eukaryotic systems, the oligosaccharide is transferred to selected asparagine residues within the -N-X-S/T- sequon of secretory proteins by oligosaccharyltransferase (OST). In eukaryotes, protein-bound oligosaccharides can be trimmed and elongated within the secretory organelles in a species-specific manner. The work of St.Geme and co-workers led to the discovery an alternative pathway for N-linked protein glycosylation in bacteria. Here, glycosylation of proteins occurs in the cytoplasm and nucleotide-activated monosaccharides serve as substrate for the N-glycosyltransferase (NGT). After glycosylation, modified proteins, predominantly adhesins, are translocated and displayed at the surface of the bacterial cell. Most interestingly, NGT glycosylates primarily the -N-X-S/T- sequon of proteins, similar to the classical OST, but the two enzymes belong to two different classes of glycosyltransferases (GT-B type, GT 41 family for NGT, GT-C type, GT 66 family for OST). Based on our detailed biochemical and phylogenetic analysis, we consider the -N-X-S/T- substrate specificity of both NGT and OST as the result of convergent evolution and propose an adaptation of NGT substrate specificity towards OST functionality. The predominant occurrence of NGT activity in mammalian pathogens suggests molecular mimicry as the selective phenotype for this convergent evolution.

(15) Structural and mechanistic studies of oligosaccharyltransferase and LLO flippase

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We investigated two essential steps in protein N-glycosylation: (i) The flipping of lipid-linked oligosaccharides across membranes (the ER membrane in eukaryotes, the plasma membrane in bacteria) and (ii) the en bloc transfer of the complex glycan from the donor LLO to the side chains of acceptor asparagines. While the first reaction is catalyzed by a flippase, the second is mediated by oligosaccharyltransferase (OST). Over the past years, we have obtained structural insight into the bacterial membrane proteins PglK and PglB from Campylobacter species. PglK is an ATP-dependent LLO flippase, whereas PglB is an OST enzyme. By developing *in vivo* and *in vitro* assays using synthetic peptide and LLO analogs, we studied various mutations of the structural elements of PglK and PglB revealed by the X-ray structures.

We determined the structures of several states of the flippase PglK, both with and without bound nucleotides. The structures revealed outward-open, outward-occluded, and inward-facing conformations. Using our *in vitro* flipping assay, we found that the inward-facing cavities of PglK did not seem to be involved in substrate (glycan, pyrophosphate moiety, or polyprenyl tail) recognition. We thus concluded that the complex LLO is directly transferred into an outward-facing and probably nucleotide-bound state of the enzyme. ATP hydrolysis seems to power the dissociation of the pyrophosphate moiety from a belt of essential arginine residues in the outward-facing cavity of the flippase. This represents a novel transport mechanism, distinct from the classical alternating-access model used for most active transport proteins.

The structure of peptide-bound PglB revealed the basis of sequon recognition and provided insight into catalytically important residues both near a bound, divalent metal ion and in a flexible loop region. The combination of structural and functional insight allowed a proposed reaction mechanism to be formulated.

In the presentation, the latest insight into PglK and PglB studies will be presented, as will preliminary results of our studies of the eukaryotic enzymes catalyzing analogous reactions.

(16) TBD

Laura Kiessling

Abstract to come

(17) Catalysis and allostery of UDP-sugar pyrophosphorylases: A new approach to anti-microbial treatments

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Allostery is the basis of a multitude of cellular signalling and regulatory processes. Because evolutionarily conserved enzymes are optimized to respond to cell- and/or system-specific needs, they differ in terms of allosteric networks. In our research we explore the links between catalysis and allostery and aim to exploit this knowledge for rational inhibitor design. Our target is the UDP-glucose pyrophosphorylase (UGP). The enzyme occupies a central position in the carbohydrate metabolism of cells from all kingdoms of life, because its product, UDP-Glc, is essential in a number of anabolic and catabolic pathways. Moreover, the significance of UGP as a virulence factor in protists and bacteria is well established and has given momentum to the search for pathogen specific inhibitors. While UGPs show high evolutionary conservation in all eukaryotes, the eukaryotic enzymes are unrelated to prokaryotic UGPs. Prominent aspects creating diversity between eukaryotic UGPs are quaternary organization and conformational flexibility. Both features are associated with catalytic functions. With the goal to understand the interplay of catalysis, allostery, quaternary organization and protein dynamics, we are using a combination of kinetic X-ray crystallography, biochemical, biophysical and theoretical chemistry. With the UGPs from *Homo sapiens* and *Leishmania major* as models, we reconstructed the enzymatic cycles. Obtained results enabled the description of structural changes during catalysis and explained the essential nature of the respective protein organisations. In our current work we extend structural and functional studies towards bacterial UGPs and the question how differences in the structure-function relationships can be exploited for specific inhibitor design. Eventually, our research shall establish a new basis on which UGPs can be exploited as drug targets.

(18) Using the chemical glycobiology toolkit to identify sensitive glycoproteins in the context of CDGs

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Defining how different CDG defects impact the glycome has immense diagnostic value but information regarding the specific glycoproteins affected and the functional consequences of this defective glycosylation is lacking. Our lab has adopted and expanded the chemical glycobiology toolkit in an effort to identify sensitive glycoproteins in the context of glycosylation-deficient cells. As a complement to metabolic engineering with modified sugar precursors, we are harnessing recombinant glycosyltransferases and modified nucleotide-sugars to selectively tag and identify cell surface glycoproteins and monitor differences in their residence. This method, referred to as SEEL (selective exo-enzymatic labeling), can be further used to identify structural differences in the cell surface glycans of normal and disease cells. The utility of this combined approach is highlighted by recent work in Cog1-deficient ldlB CHO cells. Defects in Golgi-associated COG complex subunits result in diverse glycosylation defects and cause human disease. Metabolic labeling of ldlB cells showed a profound loss of sialylation following ManNAz addition but an unexpected increase in the labeling of specific glycoproteins using GalNAz. One of the heavily GalNAz-labeled glycoproteins was identified as alpha-dystroglycan (a-DG) by proteomic analysis on the basis of its preferential loss from the cell surface following V. cholera neuraminidase treatment. Western blot analysis showed that a-DG in ldlB cells has significantly lower molecular weight compared to that of a-DG in WT CHO cells. This decrease was primarily due to simpler elaboration of the O-glycans on a-DG in ldlB cells as determined by glycomic analysis of O-glycans and SEEL-based experiments. We further showed that the underglycosylated a-DGmolecules on ldlB cells exhibit a higher rate of turnover and are more vulnerable to both bacterial proteases (including a mucinase enzyme contaminant found in V. cholera neuraminidase preparations) and to human metalloproteinases. This discovery suggests a novel function for the mucin-type O-glycans found on a-DG and highlights the power of the chemical glycobiology toolkit for identifying the most affected glycoproteins in cells with impaired glycosylation. We envision extending this experimental platform towards the study of other CDGs through the use of emerging iPSC-derived CDG cell lines.

(19) TBD

Kelly Ten Hagen

Abstract to come

(20) Consequences of genetic deficiency of heparan sulfate – What we have learned from multiple hereditary exostoses

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Multiple hereditary exostoses (MHE) is a rare disease characterized by the development of numerous benign bony tumors (osteochondromas). Patients with MHE carry heterozygous germline mutation of *EXT* genes (*EXT1* and *EXT2*), which encode a glycosyltransferase essential for heparan sulfate biosynthesis. We demonstrated that stochastic inactivation of *Ext1* in a small fraction of *Col2a1*-expressing cells using a inducible conditional knockout strategy results in the development of multiple osteochondromas and skeletal deformities in mice that closely resemble the human MHE. Jones *et al.* independently made the same observation using a different genetic mouse model. These results point to the involvement of loss of heterozygosity in the pathogenesis of MHE. Although the genetic basis of MHE has been elucidated by these studies, the cellular origin and molecular mechanisms of MHE remain elusive. We have recently found that osteochondromas develop from mesenchymal stem cell-like progenitor cells residing in the perichondrium, and that enhancement of BMP signaling in these cells is the primary signaling defect underlying MHE. We demonstrate that perichondrium-specific *Ext1* knockout mice develop multiple osteochondromas, and that Ext1-deficient perichondrial progenitor cells display enhanced BMP signaling and chondrogenic differentiation both *in vitro* and *in vivo*. Furthermore, administration of a BMP inhibitor suppresses osteochondromatogenesis in mice. Our results reveal a role for enhanced perichondrial BMP signaling in the pathogenesis of MHE, and suggest the possibility of pharmacological treatment of MHE with BMP inhibitors.

(21) ABO(H) glycans in infant heart transplantation: new insights in immunobiology and clinical applications in transplant medicine

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For organ transplantation, ABO blood group incompatibility between patients needing transplants and potential donors presents an enormous immune barrier; the need for ABO-compatibility contributes to critical shortages of donor organs and high waitlist mortality. The immune response to ABH glycans through production of 'natural' ABO antibodies mediates a rapid process of destruction upon binding to cognate non-self ABH antigens in organ transplants from incompatible donors. For heart transplantation, this hyperacute antibody-mediated rejection process would result in patient death, so crossing the ABO barrier is contraindicated. In infants, however, the developmentally delayed production of ABO antibodies allows ABO-incompatible heart transplantation to be performed successfully and safely. In the years following ABO-incompatible infant heart transplantation, B cell immune tolerance develops to the donor ABH antigens. To investigate this process and related questions, we developed a novel cross-disciplinary collaborative team "Glyconanotechnology in Translantation" between carbohydrate chemists, immunologists and clinicians. ABH structures of all 18 subtypes were chemically synthesized and used to generate a panel of monoclonal antibodies to study tissue expression of ABH subtypes in various organs and cells. Additionally, functionalized to various platforms, ABH subtype structures were used to investigate ABO immunobiology related to immunity and tolerance in patients and healthy individuals across the age spectrum from infancy to adults, and to develop new diagnostic and potentially therapeutic applications.

(22) Extrinsic glycan modeling by extracellular sialyltransferase ST6Gal-1. Potential biologic roles.

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Sialylation is generally regarded as a cell-autonomous process occurring as nascent glycoconjugates transit the intracellular secretory network. However, there are significant reservoirs of extracellular glycosyltransferases. Recently, we demonstrated the extracellular pool of the sialyltransferase ST6Gal-1 extrinsically modifies hematopoietic stem and progenitor cells within the bone marrow and acts as a systemic regulator of hematopoiesis. We also showed that platelets, upon activation, release sufficient quantities of sialic acid donor substrate to drive efficiently extrinsic sialylation. Here we present evidence that extrinsic sialylation by systemic sialyltransferases also occurs in the periphery beyond the confines of the bone marrow niche. We show that extra-medullary extrinsic sialylation, triggered by platelet activation or thrombotic events, remodels leukocyte cell surfaces in the periphery, particularly granulocytes, macrophage, and other myeloid lineage subsets, and alters the progression of inflammatory and immunological responses of the leukocytes. Potential biologic consequences of extrinsic glycoslylation will be discussed.

(23) I-branched N-glycans Negatively Regulate Melanoma Growth and Insulin-like Growth Factor Receptor Signaling

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Aberrant glycosylation plays an important role in key steps of malignant transformation and cancer progression. However, how glycan-forming and glycan-degrading machinery contribute to malignancy is still ill-defined. Here, we performed a comprehensive genomic inquiry on glycome-related genes using publically-available cohorts of nine different cancer phenotypes. We found that malignant melanoma, in particular, exhibited a glycomic gene enrichment pattern that was significantly distinct from eight other malignancies, leading to the hypothesis that melanomas are critically addicted to their glycomic nature for malignant progression. Markedly, when compared with normal human epidermal melanocytes (NHEM), this glycome gene blueprint implicated that the I-branching ß1,6 N-acetylglucosaminyltransferase 2, GCNT2, was inversely correlated with malignant transformation. Moreover, MALDI-TOF mass spectrometry on cell surface glycans revealed that NHEMs mainly expressed I-branched structures, while metastatic melanoma cells variably expressed both i-linear and I-branched modified N-glycans. With regards to its role in malignancy, enforced GCNT2 expression and subsequent cell surface I-branched glycan formation significantly decreased primary melanoma growth *in vivo*, whereas knockdown of GCNT2 expression and subsequent cell surface i-linear glycan formation significantly enhanced melanoma growth *in vivo*. Functional assessments revealed that N-glycan-dependent I-branching on Insulin-like growth factor receptor (IGF1R) decreased receptor tyrosine phosphorylation as well as *in vitro* melanoma cell growth in response to insulin-like growth factor 1 (IGF-1). Overall, these findings provide a new prospective on the importance of i/I-antigen modifications on N-glycans in melanoma development and growth factor receptor signaling.

(24) The role of the glycoproteome alterations in gastric cancer and its clinical applications

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Glycosylation alterations are frequently found in cancer and specific glycan structures are a hallmark of gastric cancer progression [1,2]. This presentation reports: (A) the increased expression of terminal sialylated structures in cancer, which can lead to the activation of tyrosine kinase receptors, such as MET and RON in gastric cancer cells [3,4,5], inducing specific intracellular signaling pathways activation and increasing cancer cell aggressive phenotypes [2]; (B) the application of glycoproteomic approaches for the identification of altered glycosylated glycoproteins, carrying the simple mucin-type carbohydrate antigen STn, in engineered cancer cell models and in sera of gastric cancer patients [6], including the demonstration that the primary gastric tumors are the source of the identified glycoproteins (CD44) expressing truncated *O*-glycans [6].

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(25) O-GlcNAc modification of Mef2c regulates C2C12 myoblast differentiation

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Unlike other types of glycosylation, *O*-GlcNAcylation is a single glycosylation occurring exclusively in the nucleus and cytosol. It is dynamically regulated by two enzymes, namely, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). Because *O*-GlcNAcylation is sensitive to changes in the levels of several nutrients such as glucose and glucosamine, it may underlie metabolic diseases, including diabetes and obesity. Furthermore, *O*-GlcNAcylation affects different ontogenic processes such as osteoblast differentiation, adipogenesis and hematopoiesis. Emerging evidence suggests that skeletal muscle differentiation is also regulated by *O*-GlcNAcylation, but the detailed molecular mechanism has not been elucidated. Moreover, the relationship between skeletal muscle differentiation and physiological condition of metabolism is not clear. In this study, we showed that hyper-*O*-GlcNAcylation in hyperglycaemic condition and hyper-glucosamine, N-acetyl glucosamine condition reduced the expression of myogenin, a transcription factor critical for terminal muscle development, during myogenesis in C2C12 myoblasts and in a mouse model of muscle injury. Because the transcriptional level of myogenin

was downregulated by the increment of total cellular *O*-GlcNAcylation, we speculated that an upstream transcription factor of myogenin might be negatively regulated by *O*-GlcNAcylation. We found that Mef2c, which binds to the MEF-binding element in the myogenin promoter, was modified by *O*-GlcNAc at Thr9, Thr 219, and Ser269. We found that myogenin expression was downregulated by *O*-GlcNAcylation on Thr9 only. Lastly, *O*-GlcNAcylation on Mef2C inhibited its DNA binding affinity. Taken together, we found that increased cellular *O*-GlcNAcylation attenuates skeletal muscle differentiation by *O*-GlcNAcylation on Mef2c, which downregulates its DNA binding affinity. Our research sheds light on the molecular mechanism involved in metabolic disease and skeletal muscle development.

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(26) Understanding and Exploiting Anti-Glycan Immunity to Improve Cancer Care

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Glycan microarray technology provides a powerful, high-throughput tool to evaluate carbohydrate-protein interactions. We have developed a glycan microarray composed of a diverse assortment of N-linked and O-linked glycans, glycolipid glycans, glycopeptides, and other oligosaccharides. Our array primarily contains neoglycoproteins as printed array components, which offers unique approaches for varying presentation on the slide surface and facile translation of discoveries to other assay platforms. Our array is currently being used in a variety of applications. First, it has been used to profile anti-glycan antibody responses induced by two cancer vaccines in late stage clinical trials, PROSTVAC-VF and GVAX Pancreas. These studies have led to new biomarkers for determining if a patient is having a productive immune response and new strategies to design more effective vaccines. Second, the array is being used to study the immunological evolution of anti-glycan monoclonal antibodies used clinically to treat cancer. These studies are providing new insights into the pathways and requirements for inducing anti-glycan antibodies. Finally, the array is being used to develop new antibodies to carbohydrates.

(27) Decoding the glycome with systems-based approaches

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Glycosylation has a vast capacity for encoding biological information and informing biological function. Our ability to decode the functional components of glycosylation in specific biological systems is attenuated by the difficulty in pinpointing specific glycans and corresponding biosynthetic enzymes involved. Systems-based approaches to biology, in which large datasets are gathered, analyzed and integrated via bioinformatic algorithms, provide an important avenue for discovering the mechanics of complex biological systems that cannot be predicted a-priori. This talk will focus on the integration of mutli'omic datasets (transcriptome, proteome, epigenome (miRNA)) with the glycome (analyzed by lectin microarray) to reveal glycans that underpin systems from melanoma metastasis to innate anitviral immunity.

(28) Molecular dissection of glycan function by simple cells and simplified tissues

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Our molecular understanding of how glycans influence cellular functions is limited. This is in part due to the complexity and technical difficulties associated with the analysis of glycans. Precise genome editing techniques now enable us to generate glycoengineered cell lines suitable for several purposes, including biopharmaceutical production, genetic dissection of glycan functions, formation of organotypic tissue models, and arraying platforms for host pathogen interactions. As the first proof-of-concept for our discovery strategy, we have used genetic engineering to deconstruct N-glycosylation in CHO cells and developed a design matrix that facilitates the generation of cells with desired glycosylation. This engineering approach will aid the production of glycoproteins with defined and homogenous glycosylation and allow genetic dissection of glycan functions in many different cells. In addition, we have pioneered a next generation approach using organotypic tissue models in combination with sophisticated mass spectrometry to decipher glycan functions. The tissue model has provided the first evidence that aberrant glycosylation in cancer directly induces oncogenic features. Moreover, the model has demonstrated that glycosylation of the envelope virus Herpes Simplex Virus 1 is essential for viral propagation. Future studies will aim to use step-by-step genetic deconstruction of glycosylation capacities and provide comprehensive cell and tissue models for discovery and dissection of structure-function relationships of glycans.

(29) B cell-Independent Antibody Sialylation

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Antibodies such as IgG are glycoproteins synthesized exclusively by B cell-lineage cells upon activation by an antigen. Recently, the anti-inflammatory benefit of high-dose intravenous immunoglobulin (IVIg) therapy in patients with severe autoimmune disease has been tied to IgG molecules

carrying terminal α2,6-linked sialic acids. This sialylated form of IgG appears to selectively downregulate the immune response and ameliorate autoimmunity. In an effort to discover the underlying regulation of IgG sialylation within the secreting B cell, we created a B cell-specific knockout of the sialyltransferase ST6Gal1, which is solely responsible for the sialic acid addition to IgG. We found that sialylation of IgG occurs independent of B cells, and that ST6Gal1 is dispensable within the B cell lineage. These mice retained the ability to sialylate IgG and circulatory ST6Gal1 activity appears to account for the sialylation event. Our findings suggest that B cells do not control the anti-inflammatory capacity of IgG, while providing strong support for an extracellular sialylation pathway within the circulatory system.

(30) New Insights in to polysaccharide capsule structure and antibody-function from Cryptococcus neoformans

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The most remarkable feature of the human pathogenic fungus Cryptococcus neoformans is a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM). The capsule is a critical virulence factor that contributes to virulence by interfering with phagocytosis and inducing a series of deleterious effects on host immunity. The capsule is poorly immunogenic but when GXM is conjugated to protein carriers it can elicit a protective antibody response. The C. neoformans capsule is a highly hydrated structure that is difficult to study but considerable insight has been gained from physical studies of the capsular polysaccharide. Using a combination of static and dynamic light scattering our group established that GXM is highly branched. Capsular enlargement appears to grow through apical extension. Analysis of antibody effects on C. neoformans capsular and soluble polysaccharide have yielded new insights into immunoglobulin function, including the observation of direct effects on fungal metabolism and the entrapment of newly released buds by sealing the capsule through crosslinking. Antibody binding to the capsular polysaccharide produces a dramatic increase in capsule stiffness, which may be an important component of its opsonic capacity. The cryptococcal capsular system is unique because of its large size relative to bacterial capsule, which allows the employment of a variety of optical and physical techniques that provide rich insights into how capsules are organized and affected by specific antibody.

(31) Carbohydrate-specific adaptive immune responses

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Glycoconjugate vaccines, which are currently made by covalently coupling a bacterial capsular polysaccharide (CPS) to a carrier protein, have been used to immunize many millions of people and have reduced disease burden, particularly among children. However, the immunogenicity and efficacy of these vaccines have been relatively low and heterogeneous in some high-risk populations. We investigated mechanisms of processing and presentation of a prototypic glycoconjugate vaccine in which a model bacterial CPS is coupled to a carrier. Upon uptake into the endosome of an antigen-presenting cell (APC), the polysaccharide undergoes oxidative depolymerization, which, in conjunction with acidic protease (e.g., cathepsin)–mediated digestion of the carrier protein, creates processed (p) carbohydrate-peptide conjugates, $glycan_p$-peptides. $Glycan_p$-peptides then bind to major histocompatibility class II (MHCII) through the peptide and are subsequently presented on the APC surface, with the carbohydrate portion recognized by the T-cell receptor of carbohydrate-specific T cells (Tcarbs). This carbohydrate recognition drives T-cell help for antibody-producing B cells. Here, we expand on our previous discovery to define the molecular mechanisms for Tcarb activation by carbohydrate epitopes from a pneumococcal glycoconjugate and a glycoprotein antigen, HIV envelope glycoprotein, gp120.

Carbohydrate antigens-what used to be known as T-cell independent antigens- can be recognized by T cells, and carbohydrate recognition by T cells may have important biomedical implications. An enhanced understanding of immune mechanisms such as T-cell recognition of carbohydrates may lead to markedly improved vaccines against infectious diseases.

(32) The story of 50,000 glycomes

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Glycans are integral component of the majority of proteins and their functional relevance is undeniable, but the knowledge about the importance of individual variation in glycosylation is still very limited. Using recently developed chromatographic, electrophoretic or MS methods we analyzed composition of total plasma and/or IgG glycomes from over 50,000 individuals. By combining glycan data with other omics, clinical and environmental data for a large number of individuals, we generated a unique tool that enables us to address some basic question about biology of protein glycosylation and its relevance in disease. For example, genome-wide association study of the IgG glycome identified 37 genetic loci that associate with variations in the IgG glycome composition. Out of these 37 loci, only four contained glycosyltransferase genes, indicating that glycosyltransferases are only a minor component of the large genetic network that regulates IgG glycosylation. Inter-individual differences in the IgG glycome composition are very large. Between 50% and 80% of this variation is heritable, while age, hormones and inflammatory processes seem to be the main confounders for the remainder of the variance. Individual variation in IgG and plasma glycome composition

can predict diseases, response to therapy and even mortality risk, which renders any clinical or epidemiological study that does not include this information seriously flawed.

(33) Efficient myelination, myelin repair and motor recovery after demyelination require Ncam1 and St8sia2

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Multiple sclerosis is a demyelinating disease of the central nervous system. Remyelination can occur spontaneously but often fails or is incomplete, most likely because of impaired differentiation of oligodendrocyte precursors into remyelinating oligodendrocytes. Polysialic acid (polySia) is a prominent posttranslational modification of mainly the neural cell adhesion molecule NCAM, which generates repulsive forces that are implicated in the control of myelination and myelin repair. PolySia is produced by two polysialyltransferases, ST8SIA2 and ST8SIA4, with overlapping but distinct cell-type expression patterns and protein acceptor specificities. Here we used the cuprizone model to study the impact of NCAM and ST8SIA2 on de- and remyelination. 8-week-old *Ncam1*$^{-/-}$ or *St8sia2*$^{-/-}$ mice and respective wildtype controls received cuprizone for 5 weeks. Despite a mild developmental delay in both, *Ncam1*$^{-/-}$ and *St8sia2*$^{-/-}$ mice, myelination at the age of 8 weeks and also the loss of myelin during the five weeks of cuprizone treatment were indistinguishable between the wildtype and knockout lines. Densities of oligodendrocyte precursors, astrocytes and microglia were unaffected. However, remyelination after cessation of the cuprizone diet was severely impaired in *Ncam1*$^{-/-}$ and, to the same extent, also in *St8sia2*$^{-/-}$ mice. This was reflected by a significant delay in the recovery of motor performance in the rotarod test. Together, these results demonstrate that NCAM and ST8SIA2 are indispensable for timely developmental myelination, efficient remyelination and motor recovery. The same negative impact of *Ncam1* or *St8sia2* deficiency indicates that remyelination requires polysialylation of NCAM by ST8SIA2. This is in stark contrast to the previously observed acceleration of remyelination in *St8sia4*$^{-/-}$ mice (Koutsoudaki *et al.* 2010 Neuroscience 171:235) revealing opposing roles of the two polysialyltransferases in remyelination and suggesting differential targeting of the two enzymes as a therapeutic strategy to improve myelin repair.

(34) C-mannosylation and its role in protein stability

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C-mannosylation is a form of protein glycosylation, where single mannose residues are covalently attached to tryptophans by unique C-C linkages. The consensus sequence for C-mannosylation is the WXXW motif, which is mainly found in thrombospondin type 1 repeats (TSRs) of different proteins and in type I cytokine receptors. Many well-known secreted and membrane bound proteins, like the four terminal components of the complement system, thrombospondin-1, and the erythropoietin receptor, have been shown to be C-mannosylated. But still, the functional role of C-mannosylation in proteins is poorly understood.

Before we identified *C. elegans* DPY-19 protein as the first active C-mannosyltransferase (1), *dpy-19* mutants of *C. elegans* worms had been described to have a characteristic dumpy phenotype. With increasing temperatures, the phenotype was shown to become more severe and finally lethal. This suggested that target proteins of the C-mannosyltransferase are increasingly dependent on their glycosylation state at rising temperatures.

Knowing the C-mannosyltransferase gene, we were able to produce target proteins with and without C-mannose in *Drosophila* S2 cells, having no endogenous C-mannosylation activity. We could show that secretion of TSR domains of *C. elegans* MIG-21 and UNC-5 increased with C-mannosylation. Interestingly, secretion of non-mannosylated domains was found to be highly impaired with increased temperature, reflecting the *C. elegans dpy-19* phenotype and suggesting C-mannosylation to influence protein stability. To further analyze this effect, a purified C-mannosylated and non-mannosylated TSR domain of UNC-5 was thermally denatured and monitored using circular dichroism spectroscopy. The C-mannosylated TSR was indeed found to be clearly more stable than the non-modified form. Also, isothermal denaturation of the TSR domain under reducing conditions was shown to occur slower in the C-mannosylated glycoform. To gain insight into stabilizing effects of C-mannosylation on a structural level, crystallization and molecular dynamic simulation studies are currently in progress.

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(35) Role of O-linked glucose modification of DNA in regulating transcription termination and gene expression in kinetoplastids

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Kinetoplastids are a group of early-diverged eukaryotes that includes the human parasites Trypanosoma brucei, and Leishmania major. The genome of these pathogens contains a modified DNA base (beta-D-glucosyl-hydroxymethyluracil) consisting of O-linked glucose modification of the thymine base such that the glucose moiety sticks out into the major groove of DNA. The modified base, called base J, is synthesized through the hydroxylation of thymidine by a

dioxygenase (JBP) forming hydroxymethyluridine, followed by the transfer of a glucose by the glucosyltransferase enzyme JGT. I will present our most recent studies on the synthesis and function of base J, including its role in regulating RNA polymerase II (Pol II) transcription termination.

The genomes of kinetoplastids are organized into polycistronic gene clusters that contain multiple genes co-transcribed from a single promoter. We have localized base J at regions flanking the polycistronic clusters at sites involved in initiation and termination and demonstrated that base J at promoter regions regulates Pol II recruitment and transcription initiation. We now show base J is also present within polycistronic gene clusters causing 'premature' termination. Reduction of J at these sites leads to readthrough transcription and increased expression of the downstream genes, including genes involved in parasite pathogenesis. Presumably, the glucose moiety of base J provides a steric block for transcription elongation stimulating termination.

Our working model suggested that the specific localization of J is regulated by the thymidine hydroxylases (JBP1 and JBP2), while the GTase is non-specific, able to add glc to hmU wherever it is located in the genome. We have now confirmed this hypothesis by identifying the base J specific GTase, JGT, and demonstrating it utilizes UDP-Glc to transfer Glc specifically to hmU in dsDNA in a sequence independent manner. Furthermore, genome-wide mapping of hmU in the JGT knock-out cell line, utilizing recombinant JGT enzyme and antibody pull-down of J-DNA fragments, indicate hmU synthesized by the JBP's is located at J specific sites of the genome seen in wild-type cells. These studies confirm the two-step pathway of J synthesis and that the specific localization of J in the genome is dictated by JBP1 and JBP2.

(36) Arabidopsis as a model system to study N-glycan-based protein quality control

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The physiological function of a given protein is determined by its structure, yet acquiring a correct three-dimensional structure is an error-prone process that often leads to accumulation of toxic misfolded proteins, a major cause of human diseases such as Alzheimer's disease and Parkinson's disease. Fortunately, eukaryotic cells are equipped with several protein quality control (QC) systems that recognize, repair, and/or remove misfolded proteins to maintain cellular protein homeostasis, including one in the endoplasmic reticulum (better known as ERQC). Most of our current knowledge on ERQC comes from biochemical and genetic studies in yeast and mammalian cells. Although ERQC is known to operate in plant cells, very little is known about its molecular components and its biochemical mechanism. Our recent studies demonstrated that the dwarf phenotype of two well-known Arabidopsis mutants, *brassinosteroid-insensitive 1-5* (*bri1-5*) and *bri1-9*, are caused by ER retention and subsequent degradation of structurally-defective yet biochemically competent brassinosteroid receptor BRI1 that functions on the plasma membrane to initiate a phosphorylation-mediated signaling cascade critical for normal plant growth and development. Forward and reverse genetic studies with these two *bri1* mutants resulted in identification of not only evolutionarily-conserved but also plant-specific components of an Arabidopsis ERQC system. The initial genetic discoveries and subsequent biochemical studies of these ERQC components in Arabidopsis will be presented at the meeting.

(37) N-glycosylation modulates the tethered-extended equilibrium of the extracellular domain of EGFR

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Introduction: The Epidermal Growth Factor Receptor (EGFR), a tyrosine kinase glycoprotein, is over expressed in several cancers. EGFR activation triggers various signaling cascades in the cytosol associated with cell proliferation and differentiation. The monomeric form of the EGFR extracellular domain is known to be auto-inhibited (tethered state) on the cell surface by extensive intramolecular contacts. Binding of growth hormone to this monomer is accompanied by removal of the intramolecular contacts, rearrangements of subdomains I, II and III of the extracellular domain (untethered state) and a back to back dimerization of EGFR. Glycosidically bonded oligosaccharides to EGFR extracellular domain contribute to this tethered-extended transition.

Objective: We used accelerated Molecular Dynamics (aMD) simulations to investigate the role of N-glycosylation on the EGFR tethered-extended transition.

Results: Dynamics of N-glycosylated EGFR in untethered and tethered state. Tethered and untethered EGFR showed more restricted motions upon glycosylation. The narrow range of sampled conformations around the initial structures supports the suggested stabilizing role of glycans. Glycosylation supports the untethered state and weakens tethering interactions. EGFR when in its tethered state, remains stable in both its unglycosylated and glycosylated forms. However, when untethered, the unglycosylated state tends to adopt a compact conformation which is characterized by proximity of subdomain IV to the dimerization arm, and is similar to the tethered state (as seen in the PC plots); upon glycosylation, untethered EGFR remains untethered. The untethered state of EGFR is obtained by the removal of the interactions between the dimerization arm and the two inhibitory loops of subdomain IV. The distance between the center of mass of each inhibitory loop and the dimerization arm increases upon glycosylation.

Conclusions: N-glycosylation maintains the extended state of monomeric EGFR. N-glycosylation weakens intramolecular tethering interactions and supports the transition from the tethered to the untethered conformation. In the absence of attached oligosaccharides the untethered conformation of monomeric EGFR could adopt a tethered conformation.

(38) Maturation of Asn-linked glycans in the mammalian secretory pathway: structural basis of substrate recognition by GH47 alpha mannosidases

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Maturation of Asn-linked oligosaccharides in the eukaryotic secretory pathway requires trimming of the nascent glycan chains to remove all glucose and several mannose residues prior to extension into complex type structures present on cell surface and secreted glycoproteins. Multiple GH47 alpha mannosidases, including endoplasmic reticulum (ER) alpha mannosidase I (ERManI) and Golgi alpha mannosidase IA (GMIA), are responsible for cleavage of the alpha 1,2-linked mannose residues on the glycan chains. These cleavage steps produce uniquely trimmed oligomannose isomers that are necessary for ER glycoprotein quality control and glycan maturation. ERManI and GMIA have similar catalytic domain structures, yet they cleave distinct residues from tri-branched oligomannose glycan substrates. The structural basis for enzyme-substrate interactions and cleavage by ERManI and GMIA was explored initially through exchange of an essential enzyme-bound Ca^{2+} ion with La^{3+}. This ion swap led to enzyme inactivation, but maintained high substrate binding affinity. Co-crystallization with $Man_9GlcNAc_2$ substrate analogs revealed enzyme-substrate complexes with distinct modes of glycan branch insertion into the respective enzyme catalytic pockets. Both enzymes had glycan interactions that extended from a core GlcNAc residue to the terminal mannose residue being cleaved, but they engaged different glycan branches and employed different sets of glycan interactions. Enzyme-glycan interactions were also probed by mutagenesis, time course analysis of glycan cleavage, and kinetic analysis. The resulting data indicate that glycan maturation in the secretory pathway is governed by steric access to the full oligosaccharide structure, which determines the efficiency of mannose trimming reactions and subsequent conversion to complex type structures in mammalian cells.

(39) Glycoproteomic analysis of human glycoproteins in STT3A(-/-) and STT3B(-/-) knockout cell lines

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Mammalian cells express two oligosaccharyltransferase (OST) complexes that contain different catalytic subunit (STT3A or STT3B) assembled with a shared set of accessory subunits and one to two complex specific subunits. The STT3A complex is responsible for cotranslational glycosylation of nascent proteins as they enter the ER lumen while the STT3B complex can mediate posttranslocational glycosylation of acceptor sites that have been skipped by the STT3A complex. Expression of both OST complexes in human cells is necessary to achieve full glycosylation of the N-glycoproteome.

We have used the CRISPR/Cas9 gene editing technology to create viable HEK293 derived cells lines that are deficient for a single catalytic subunit (STT3A or STT3B) or two STT3B-specific accessory subunits (MagT1 and TUSC3). Initial pulse-chase labeling of a panel of glycoproteins that contain previously characterized STT3A and STT3B dependent glycosylation sites revealed more severe glycosylation defects in HEK293 knockout cell lines, than in cells treated with siRNA. We used a glycoproteomic approach that includes stable isotope labeling with amino acids in cell culture (SILAC) coupled with lectin affinity enrichment for glycopeptides followed by detection by high-resolution LC MS/MS. Glycosylated peptides from STT3A(-/-) cells and STT3B(-/-) cells were quantitatively compared with peptides from wild type cells. Our analysis resulted in a dataset with ~1250 quantified glycosylation sites in 600 glycoproteins. Significantly underglycosylated sites represented 27% and 50% of the total quantified sites for the STT3B(-/-) and STT3A(-/-) cells, respectively. Between 5 and 10% of sites were hyperglycosylated in STT3B(-/-) or STT3A (-/-) cells. Two major classes of STT3B-dependent sites could be defined based on our glycoproteome dataset: extreme C-terminal sites that are located within 60 residues of the C-terminus, and sequons located in the loops of polytopic membrane proteins with more than 5 transmembrane spans. Additionally, we found that hypoglycosylated sites in STT3B (-/-) cells have an increased proportion of bulky or acidic residues at the X position of the sequon (NXS/T) relative to sites that did not show hypoglycosylation. Currently, we are using pulse-chase labeling procedures to verify altered glycosylation of a subset of the hypoglycosylated and hyperglycosylated proteins.

(40) Presence of multiple isomers of polygalactosylated-Fucose (polyGaln=1-6-Fuc) containing high-mannose and paucimannose type N-glycans in planaria S.mediterranea

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Carbohydrate moieties, attached to proteins and lipids on cell surfaces, display a characteristic cell-specific expression during different stages of development and differentiation. The variations in glycan profile during differentiation of ESCs and iPSCs suggest that glycans play a pivotal role in cell fate decisions. However, little is known about the molecular mechanisms involved in their role in maintenance

of cellular pluripotency and regeneration. The robust regenerative ability of planarians has made it the ideal model system to explore the properties of stem cells and delineate the precise mechanisms in regeneration. With the availability of a tractable model system such as planaria, the role of glycans in tissue regeneration and homeostasis was studied. Ultrasensitive MALDI-TOF MS analysis of permethylated N-glycans revealed that S.mediterranea contains high-mannose, paucimannose, and complex type N-glycans in decreasing order of abundance. Additionally, N-glycans of m/z 1753, 1987, 2162, 2366, 2570, and 2774 composed of $Fuc_1Hex_{n=5\text{-}10}HexNAc_2$, which differed by one hexose unit, were also observed. The b- and y-ions obtained in MALDI-TOF/TOF MS^2 analysis of permethylated N-glycans revealed that these masses were composed of multiple isomers of polygalactosylated(n=1-6)-Fucose attached to GlcNAc at the reducing end and mannose (n=3-9) at the non-reducing end. The presence of galactose was confirmed by enzymatic digestion and GC-EIMS. Given that core fucosylation is a common addition, its distribution was determined by whole-worm lectin histochemistry using fucose-specific lectin *Aleuria aurantia* (AAL). Fucosylated N-glycans was found to be specific to ventral cyanophilic gland cells and their secretory projections. Macrophage mannose receptor, C-type lectin 'collectin', and matrix endopeptidase were identified as the target proteins for fucosylation through nano LC-MS/MS analysis of proteins obtained by affinity chromatography on AAL-agarose. Interestingly, while Gal-Fuc has been reported in invertebrate systems, polygalactosylation and existence of multiple isomers have not been observed in other organisms. Polygalactosylation could possibly be mediated via the activity of enzyme GalT-1 that transfers galactose to core fucose. The presence of novel isomers indicates that a new biosynthetic pathway could be at play. The selective distribution of fucosylated glycans confirms the cell-specific distribution of glycans that can be exploited for identifying specific cell types and determining their physiology.

(41) N-GLYCOME PROFILE IN MEDAKA FISH EXPOSE TO LOW DOSES OF IONIZATION RADIATION

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Ionizing radiation (IR) is released by atoms in the form of electromagnetic waves or particles. While low dose IR has numerous beneficial applications, it has the potential to increase the risk of detrimental long term health effects. However, the real risk of low dose IR on health is still unclear. A glycomic marker may prove to be a promising tool to monitor organismal changes that result from IR exposure. N-linked glycosylation occurs through a highly conserved process, and affects many properties of proteins including conformation, solubility, and antigenicity. N-glycans play important roles in cell signaling and the immune response. Defects in synthesis may result in a variety of human diseases. Medaka is emerging as an important model organism and provides a useful tool to study changes in the proteome and glycome when exposed to different levels of IR. Medaka were irradiated with 0.1 mGy, 2mGy, 20mGy and 200 mGy IR for 6 months to allow for chronic accumulation. After exposure, fish were dissected into three tissue types: carcasses (including eyes and brain), organs, and intestines. N-glycans were obtained by enzymatic hydrolysis using PNGase F followed by MALDI-TOF and ESI-MS, and bioinformatic analysis. To date, results from carcasses have shown significant differences in expression as a function of IR. A difference in response with increasing dosage in all treatments could be observed. Specifically, in the highest dose treatment the number of signals in the spectra decrease in comparison with the other samples. Four glycans, at 2373, 2769, 2973 and 3177 m/z, were shown to demonstrate sensitivity to radiation. Based on searches of the CFG database, their m/z correspond to complex or hybrid glycans, three containing fucose. However, the corresponding structures are still not fully elucidated. The detailed structural elucidation and roles of other glycans related to low dose radiation are under investigation. Additionally, certain N-glycans were unique in one treatment, and could be potentially useful biomarkers. These results are just the first step in our search for a biomarker for low dose radiation.

(42) TREX1 regulates oligosaccharyltransferase to prevent the liberation of bioactive atypical free oligosaccharides and autoimmune diseases

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TREX1/DNase III is an endoplasmic reticulum (ER) tail-anchored exonuclease whose mutations are associated with several autoimmune and inflammatory diseases. Mutations that disrupt the N-terminal DNase domain causes chronic activation of innate immune response to self-DNA. In contrast, frame-shifts (fs) mutations located at the C-terminus alter TREX1 localization, without affecting TREX1 DNase function. We recently showed that the TREX1 C-terminus interacts with the oligosaccharyltransferase (OST) subunits RPN1 and DDOST, and that TREX1 disease-associated fs mutants dysregulate OST's preference for LLO hydrolysis to generate bioactive free oligosaccharides in the cell. Here, we further define the structure and immunogenicity of free oligosaccharides generated in Trex1-/- cells. Structural analysis on Trex1-/- free oligosaccharides revealed that they differ from typical N-glycans and that they potentially resemble paucimannose structures. Using the newly established TREX1-V235fs mouse model, we found that a broad panel of IgG and IgM autoantibodies are significantly elevated in TREX1-V235fs mouse serum

compared to wild type. We also observed elevated anti-glycan antibodies. Together, we propose a molecular mechanism where TREX1 fs mutations cause accumulation of bioactive free oligosaccharides, which trigger immune activation and autoantibody production leading to immune disorder.

(43) Identification of novel transporters for UDP-arabinose in plants

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Arabinose is one of the most abundant sugars in glycoproteins and polysaccharides in plant cell walls. Most of the arabinose is present in the furanose form rather than the pyranose form. This presents a conundrum since de novo synthesis of UDP-Ara*p* takes place inside the Golgi lumen by epimerization of UDP-Xyl, whereas the conversion of UDP-Ara*p* to ADP-Ara*f* is catalyzed by a mutase located in the cytoplasm. This indicates that UDP-Ara*p* must be transported out of the Golgi lumen and UDP-Ara*f* must be transported into the Golgi lumen, where the arabinofuranosyltransferases reside. Using a novel approach that combines reconstitution of NSTs into liposomes and subsequent LC-MS/MS analysis of nucleotide sugar uptake we identified a small family of six nucleotide sugar transporters in Arabidopsis that are specifically transporting UDP-Ara*f*. None of the six transporters show significant transport activity with any of the 13 other nucleotide sugar substrates tested. UDP-Ara*f* concentration in the cytoplasm is low compared to most other nucleotide sugars, but four of the transporters have a low Km for this substrate. Two of the transporters have a higher Km suggesting that they may transport another nucleotide sugar than UDP-Ara*f*. Transient expression of the UDP-Ara*f* transporters in tobacco confirms that they are located in the Golgi apparatus. Data obtained from mutant and overexpression lines of Arabidopsis indicate that the import of UDP-Ara*f* from the cytosol into the Golgi lumen is a critical step in cell wall biosynthesis and important for plant development. Identification of the four UDP-Ara*f* transporters adds to the 11 other nucleotide sugar transporters in Arabidopsis that we have previously reported.

(44) Regulation of protein O-glycosylation in epithelial cells – the polypeptide GalNAc-transferases direct cellular differentiation and maintenance of tissue homeostasis

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Posttranslational modifications (PTMs) diversify protein functions and greatly expand the potential for regulation of protein activity, with O-glycosylation being among the most abundant and diverse PTMs. Initiation of O-GalNAc glycosylation is regulated by 20 distinct GalNAc-transferases (GalNAc-Ts) with somewhat overlapping functions *in vitro*. Deficiencies of individual GalNAc-Ts produce cell- and protein-specific effects resulting in subtle distinct phenotypes in insect and murine models. Yet, we have little understanding of the cell- and tissue-specific functions of the individual GalNAc-Ts. We have recently used precise genetic engineering to target the human C1GalT1 chaperone COSMC to generate stable cells and tissue models with homogenous truncated GalNAc O-glycans, which allowed us to characterize the functional consequences of simplified O-glycosylation in tissue formation. We now extend these studies to characterize the impact of site-specific glycosylation on epithelial differentiation. Using an organotypic model system equipped with cells with and without three GalNAc-Ts, we identify distinct phenotypes associated with the loss of the individual transferase activity, which is substantiated by changes in expression patterns of known differentiation markers and adhesion molecules. Furthermore, we find non-redundant O-glycosylation performed by single GalNAc-Ts using quantitative differential analysis of O-glycoproteomes and identify isolated sites of O-glycosylation in critical positions on numerous proteins. To further investigate the impact of site-specific O-glycosylation on cellular differentiation and the pathways involved in epithelial differentiation, we performed differential transcriptomic RNAseq analysis of HaCaT cells with or without the three GalNAc-Ts, revealing changes well-correlated with the observed phenotypes. Altogether, we find that the individual GalNAc-Ts participate in the formation of human epithelia and selectively affect distinct cellular pathways. Further investigation of isoform specific substrates in the context of identified cellular pathways could explain the observed phenotypes in tissue formation.

(45) T Cells require extended O-glycosylation to populate peripheral lymphoid organs.

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T cells express a wide variety of glycoproteins for adhesion, signal transduction, and effector functions. Global glycosylation of T cells changes with the differentiation stage and activation state, but the role of glycosylation in T cell function is not well understood. To explore this role, we used targeted gene deletion to generate mice with T cells lacking the essential chaperone Cosmc, without which cells cannot create extended O-glycans. These mice had dramatically reduced

numbers of T cells in their blood and peripheral lymphoid organs, and the T cell population was dominated by cells that had escaped gene deletion. However, thymic populations were undiminished; knockout cells were present, and appeared to develop normally through the single positive stage. Adoptive transfer of CD4 single positive thymocytes into wild type animals showed that knockout T cells had a reduced ability to populate peripheral organs and were rapidly cleared from circulation. These results demonstrate that extended O-glycosylation of T cells is crucial to their ability to migrate into lymphoid organs and populate the periphery.

(46) Systems Biology of Caenorhabditis elegans Glycosyltransferases.

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Glycosyltransferases catalyze the transfer of glycosyl groups from sugar substrate donors (containing a nucleotide phosphate or a lipid phosphate leaving group) to a host of acceptor substrates – oligosaccharides, monosaccharides, proteins, lipids, nucleic acids, and other small molecules; effectively GTs catalyze glycosidic bond formation between these multifarious possibilities of molecules. Numerous studies show that GTs are important for developmental and physiological processes in *C.elegans* (Berninsone, 2006). However, several GTs have not yet been empirically validated. *C. elegans* utilizes GTs for critical functions, including pheromone signaling with ascarosides and the detoxification pathways, though identities of GTs regulating these functions remain unknown.

C. elegans controls its behavior and development through the use of ascarosides, which are also present in many free-living and parasitic nematodes. Some of the phenotypic manifestation of the secretion of ascarosides include aggregation, olfactory plasticity, dauer formation, attraction behavior, and hermaphrodite behavior. (Srinivasan *et al* 2008; Ludewig & Schroeder, 2013) Chemically, ascarosides are defined as glycosides of dideoxysugar ascarylose, attached to a fatty acid side chain, thus implicating GTs in its biosynthesis. In addition, the innate immune system in *C. elegans* utilizes a wide range of immune effectors and enzymes (including GTs) for microbial defenses and xenobiotics detoxification. (Lindblom & Dodd, 2006; Stupp *et al* 2012) Stupp *et al* 2012 showed that *C. elegans* can detoxify two bacterial toxins, 1-hydroxyphenazine (1-HP), and indole via N- and O-glycosylation. Our research aims to discover the roles of specific GTs in specific biological processes like these.

In this exploratory study, we selected a dozen GT mutant strains, majority of which belong to the GT-A fold protein (families 2, 7, 21, 27, and 13). The goal is to study the function of these GTs using a systems biology approach in order to construct models for GTs functions using the following omics platform – glycomics, metabolomics, phenomics, and transcriptomics. For GTs with unavailable mutant strains, we are carrying out knock-out experiments using the homology-directed genome editing via CRISPR-Cas9 ribonucleoprotein complexes (Paix *et al*, 2015).

Here, we will show some of our preliminary phenotypic and global metabolomics data.

(47) A Campylobacter jejuni bacteriophage depends on early pseudaminic acid biosynthesis enzymes for infection

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Campylobacter jejuni is an excellent model organism for studying bacterial protein glycosylation since unlike most other bacteria, it has both N- and O-linked protein glycosylation systems. While *C. jejuni* N-glycosylates >60 proteins, its O-glycosylation system only glycosylates the flagellar FlaA and FlaB subunits, and this modification is essential for filament assembly. Each flagellin monomer is glycosylated up to 19 times with sialic acid-like nonulosonic acids such as pseudaminic acid (Pse), legionaminic acid and their derivatives, resulting in approximately 10% of the protein mass being contributed by glycans. CMP-pseudaminic acid is synthesized in the cytoplasm from UDP-N-acetylglucosamine through a six-step conversion involving the enzymes PseB, PseC, PseH, PseG, PseI and PseF, all of which are required for flagellar filament assembly.

Though bacteriophages are increasingly appreciated for their roles in shaping bacterial lifecycles, phage interactions with bacterial protein glycosylation systems have not been described. We have observed that the *C. jejuni* phage NCTC 12673 depends on early-acting enzymes of the *C. jejuni* Pse biosynthesis pathway (PseBCH) to successfully infect cells. However, any late-acting enzymes (PseGIF) can be knocked out without affecting infectivity.

PseH, an acetyltransferase, catalyzes the conversion of UDP-4-amino-4,6-dideoxy-β-L-AltNAc to UDP-2,4-diacetamido-2,4,6-trideoxy-β-L-altropyranose (intermediate IV). After this step, PseG removes UDP, leaving 2,4-diacetamido-2,4,6-trideoxy-β-L-altropyranose. The apparent importance of the UDP-activated glycan implicates a role for intermediate IV in protein or nucleic acid glycosylation. We hypothesize that one of three phenomena is occurring: 1) phage assembly requires protein(s) to be glycosylated by intermediate IV, 2) phage DNA requires glycosylation by intermediate IV, or 3) cells with defects early in the Pse pathway signal a stress response that restricts phage infection.

We analyzed phage deoxynucleosides by mass spectrometry and found no evidence of DNA glycosylation. We are currently analyzing the phage proteome for glycopeptides

and the transcriptome of phage-infected wild type, *pseC* (early)- and *pseF* (late)-mutant cells. This should point to the stage at which phages are inhibited during infection of an early mutant (ie. host shutdown, DNA replication and/or assembly) while also identifying differences in cellular gene expression between early and late *pse* mutants that could explain the observed phenotype.

(48) Increased susceptibility to ionizing radiation in mice with ST6Gal-1 deficiency

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Sporadic reports have associated the degree of N-linked α2,6 sialylation, mediated by the sialyltransferase ST6Gal-1, with increased chemo- and radio-resistance of tumor cells, therapeutic failures in colorectal cancers, and protection against Fas-mediated apoptosis. Radiation exposure, whether iatrogenic or accidental, is a significant health concern. ST6Gal-1 has been suggested as a candidate biomarker for exposure to ionizing radiation; both enzyme expression and the level of protein sialylation increase following radiation exposure in a dose dependent manner. Here we report a striking sensitivity of animals with non-functional ST6Gal-1 to ionizing radiation. We attempted to rescue st6gal1-KO and wild-type C57BL/6J mice that were subjected to 12 Gy full body irradiation by bone marrow transplantation with congenic wild-type marrow cells. While the wild-type, st6gal1-normal animals uniformly survived the procedure, st6gal1-KO mice suffered 100% morbidity within 8 days of irradiation. There was comparable homing and retention of donor cells to the marrow of st6gal1-KO and wild-type recipients, and failure of hematopoietic cell engraftment was unlikely as a cause for morbidity. However, st6gal1-KO recipients suffered heavy damage to the gastrointestinal tract resulting in an almost complete loss of absorptive tissue and integrity of the gastrointestinal architecture. This damage was far less severe in the wild-type recipients. Damage to the gastrointestinal tract is an especially frequent and detrimental complication of radiation used in cancer therapy. Our data indicate a critical role for ST6Gal-1 in the maintenance of radioresistance in the gastrointestinal tract. (This work was supported by the National Program of Excellence in Glycosciences grant P01HL107146 and grant R01AI056082.)

(49) Platelet derived sialic acids support extrinsic sialylation in vivo

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Sialylation is generally regarded as an intracellular process occurring as nascent glycoconjugates transit the Golgi-ER secretory network. However, significant reservoirs of extracellular glycosyltransferases are present in systemic circulation. Recently, we demonstrated the alpha 2,6 sialyl structures on hematopoietic cells can be constructed by extracellular, systemic ST6Gal-1, and this pool of extrinsic ST6Gal1 acts as a systemic regulator for granulopoiesis. We also showed that platelets have sufficient sialic acid stores to drive extrinsic sialylation *in vitro*. Here, we provide direct visualization that activated platelets do participate in extracellular sialylation *in vivo* by supplying sialic acid donor substrates. CMAH -/- mice, which lack the hydroxylase that produces Neu5Gc, received transfusions of Neu5Gc-containg platelets from C57/Bl6 mice. We tracked the attachment of Neu5Gc, originating only from the transfused platelets, onto the natively Neu5Gc-negative CMAH -/- cells. Three hours after eliciting acute airway inflammation by intratracheal LPS administration, cell surface Neu5Gc on airway leukocytes as well as circulatory CD11b+Ly6G+ neutrophils was readily observed. Transfer of platelet-originated Neu5Gc onto host cells was not detected at baseline in the absence of the LPS insult. We reasoned that LPS-induced inflammation has concomitant components of platelet activation and thrombosis. To disengage these events, the FeCl3 model of mesenteric artery thrombosis without inflammation was examined to evaluate the requirement of inflammation in extrinsic sialylation. Within ten minutes after application of FeCl3, thrombotic clots were readily observed on the arterial wall, with NeuGc labeled endothelial cells clearly visible. Together, the data furthers the notion that extrinsic remodeling of target cells by sialylation is physiologically relevant, and that platelet activation is an important checkpoint regulating extrinsic sialylation *in vivo*. (*This work was supported by the National Institutes of Health Program of Excellence in Glycosciences grant P01HL107146 and grant R01AI056082*)

(50) Identification of glycosylation sites and mutations determining antigenic drift events for influenza A viruses using sparse group lasso regression

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Influenza is responsible for a substantial disease burden worldwide and vaccination remains the primary strategy for its prevention and control. The subtype H1N1 influenza A virus (IAV) caused two documented pandemics in 1918 and 2009 as well as the seasonal outbreaks since 1977. The mutations in surface glycoproteins of IAV, especially hemagglutinin (HA) can lead to biophysical and structural changes in the antibody binding sites either through differences in biochemical properties of amino acids and glycosylation patterns, potentially leading to drift of viral antigenicity thus decrease or even loss of the vaccine efficiency. Identification of key residues in influenza genomic sequences will be important to develop sequence based

vaccine strain selection method. In this study, a novel and sparse learning based computational model for feature selection was developed. This method introduces a bootstrapped sparse group lasso regression to handle three different groups of feature (mutation, N-glycosylation site and O-glycosylation site) on the HA1 of H1N1 IAVs. This method was applied and validated in H1N1 seasonal IAVs (1977-2009) and 2009 H1N1 IAVs (2009-2015), including 2,649 pairs of H1N1 seasonal IAV specific HI titers from 274 viruses and 79 sera and 2,947 pairs of A(H1N1)pdm09 specific HI titers from 413 viruses and 65 sera. Results showed that, in addition to mutations, the changes in both O-linked and N-linked glycosylation could have contributed to antigenic drift for H1N1 IAVs. Compared to those derived from mutations, changes in the potential glycosylated sites had higher impacts on the antigenic changes. In addition, the model incorporating glycosylation information outcompeted that with mutations only. In summary, using the residues and glycosylation sites on HA1 protein, which were selected by the sparse group lasso model, the proposed model could predict the antigenic variant and drift. This method could be helpful for vaccine strain selection.

(51) The Gut-Brain Axis: a glycoproteomic view

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The Gut-Brain axis is a bidirectional communication system that integrates neural, hormonal and immunological routes to properly maintain gastrointestinal homeostasis, digestion, the immune system, mood, behavior and cognition. Increasing evidence show that gut-microbes can communicate with the brain and modulate brain development, function as well as behavior through mechanisms that are yet unclear. Interactions of microbes with host-glycans and glyco-conjugates on the cell surface of epithelial intestinal cells and neural cells are poorly understood mainly due to the lack of structural knowledge of host-glycans and glyco-conjugates. We developed a glycoproteomic approach to i) obtain detailed profiles of host cell-surface glycans along the gastrointestinal tract and in different functional brain areas in mice; ii) identify corresponding host cell-surface glycoproteins; and iii) to determine the site-specific glycoprotein occupancy. The cell surface N-glycome of gastrointestinal tract and brain areas including forebrain and hindbrain were obtained from P21 and adult mouse by nano liquid-chromatography-time of flight mass spectrometric analysis (nanoLC-CHIP-TOF/Q-TOF-MS). LC-Orbitrap-MS analysis of cell surface proteins obtained from adult mouse forebrain areas (cortex, hypothalamus, and hippocampus) allowed for the identification of more than 3100 proteins, 1600 of which were plasma membrane glycoproteins. Enrichment and analysis of glycopeptides derived from hippocampus rendered the identification of more than 200 glycopeptides and permitted the assignment of more than 150 cell-surface glycoprotein site–specific occupancy. The results obtained suggest the complexity of host cell surface glycans and glycoproteins in the gut-brain axis, that can serve as targets for interactions with gut microbes and participate in the microbiota-gut-brain axis cross talk.

(52) Involvement of glycosylation and proteasomal protein degradation in O2-dependent development in Dictyostelium

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Low oxygen conditions inhibit ability of the social amoeba *Dictyostelium* to complete its fruiting life cycle, which may ensure its occurrence above ground. A possible mechanism involves the Skp1-Cullin-Fbox (SCF) family of E3 ubiquitin ligases. The SCF and proteasome are essential in eukaryotes for the regulation of many cellular processes through turnover of specific substrates. In *Dictyostelium*, PhyA (an orthologue to the human oxygen sensor) hydroxylates and therefore promotes the glycosylation of Skp1. This O_2 rate limited glycosylation has been shown to increase the degree of Skp1's binding to 3 different Fbox proteins, JcdI, FbxD and FbxA. Increased Fbox binding may alter substrate stability and influence the cell's ability to regulate its proteome. In effect, variable oxygen levels can alter the proteome of the cell through alterations in activity of the SCF.

A *phyA* knockout (*phyA*$^-$) have been used to simulate a low oxygen environment and, as expected, the mutants have difficulty developing past the slug stage. Consistent with the idea that glycosylation increases Skp1's interaction Fbox proteins, *phyA*$^-$ cells appear to have reduced SCF activity as shown by lower background levels of poly-K48-ubiquitinated proteins.

Proteasomal degradation appears to be important for development based on the inhibitory effects on developmental timing and spore generation by 40-80 μM MG132, a specific inhibitor of the 26S proteasome. These effects correlate with a two-fold increase in K48-linked poly-ubiquitylated protein. Remarkably, in *phyA*$^-$ cells, MG132 treatment partially rescued the ability of *phyA*$^-$ cells to fruit based on morphology and spore numbers. The fruiting coincides with the rescue of the K48-polyubiquitinome of the cells relative to wild-type cells.

Furthermore, higher levels FbxD and JcdI have been observed in *phyA*$^-$ slugs despite significantly lower levels of Skp1 association. Since Skp1 modification appears to

promote SCF assembly, a possible explanation is regulation through auto-ubiquitylation of Fbox proteins as described in other systems. Future studies will search for culmination factors that are enhanced by proteasome inhibition and examine how they relate to oxygen sensing by the SCF.

(53) Cell Non-Autonomous Regulation of Neural Sialylation

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Sialic acids commonly occupy the terminal position on glycan chains playing pivotal roles in many biological functions. Sialylation is known to be essential for mammalian neural development, but its role in neural excitability is less understood. Studies of neural sialylation in mammalian organisms are often hindered by the complexity of the nervous system and glycosylation pathways. Drosophila shares many of the genes involved in the biosynthesis of sialoglycoproteins, making it a convenient model to uncover evolutionarily conserved mechanisms of sialylation-mediated control of neural excitability. Drosophila sialyltransferase, DSiaT, is closely related to ST6Gal-II in mammals. ST6Gal-II is enriched in the nervous system of mice, yet its biological function is still unknown. We have shown that both DSiaT and CMP-Sialic Acid Synthetase (CSAS), the enzyme responsible for the penultimate step in the sialylation pathway, are expressed in the nervous system where they function in a pathway that regulates neural excitability. We demonstrated that Drosophila CSAS is highly conserved and that its human counterpart, hCSAS, could rescue the phenotype of Drosophila mutants lacking endogenous CSAS, despite their dissimilar localization within the cell. Our current project focuses on the regulatory mechanisms that control neural sialylation. We examine the cell-specific requirement of sialylation using behavioral assays, electrophysiology and immunostaining. We apply a rescue strategy using the UAS-GAL system with cell specific drivers. Our results suggest that CSAS is required in glial cells for proper maintenance of neural transmission. Furthermore, immunostaining shows that CSAS is expressed in glial cells, while DSiaT is present in neurons, suggesting that CSAS can affect neural functions via a cell non-autonomous mechanism. Our data suggest that CSAS represents a regulatory bottleneck of the pathway and participates in neuron-glia coupling. We currently investigate possible cellular and molecular scenarios that could underlie regulation of neural sialylation, including intercellular transport of nucleotide sugar substrates and extracellular remodeling of glycan chains. In summary, our results uncovered a novel bipartite regulation of neural sialylation in Drosophila. These data could shed light on analogous, evolutionarily conserved regulatory mechanisms that control sialylation in mammalian organisms. This project was supported in part by the NIH/NS075534 grant to VP.

(54) Chemistry based tools to explore tyrosine O-glycosylation

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Protein *O*-glycosylation on Thr and Ser residues are of great impact in key biological functions. In 2011, two independent groups (Larsson/Nilsson and Clausen lab) [1-2] described that mucin type *O*-glycosylation also modify Tyr residues, a modification long time overseen. In a later glycoproteomic study by the Medzihradszky group, mitochondrial glycopeptides were additionally identified with Tyr glycosylation.[3] Due to lack of proteomic tools that differentiate between GalNAc and GlcNAc residues, the study was not able to confirm if the *O*-glycosylations were of mucin type or *O*-GlcNAcylation. In 2015, the Hart group described that numerous mitochondrial proteins are densely modified by O-GlcNAcylation, including nearby sites of the previously identified HexNAc-Tyr modified glycoproteins.[4] This knowledge together with the sub-cellular localization and existence of competing Tyr phosphorylation sites, makes it likely that the identified mitochondrial peptides were modified with β-*O*-GlcNAcylation. However, until now β-*O*-GlcNAcylation on Tyr is not described as a known modification since current methods were not able to prove its identity.

Construction of molecular tools enabling chemical biology studies of GalNAc- and GlcNAc-Tyr modifications are here described in more detail. In a publication from last year, we showed that the MS-fragmentation patterns of synthetic peptides and tryptic peptides, differ between β-GlcNAc- (*O*-GlcNAcylation) and α-GalNAc- (mucin type) glycans attached on Thr or Ser residues.[5-6] Using synthetic glycopeptides, we confirmed that these differences in oxonium-ion MS-fragmentation also apply for Tyr *O*-glycosylation. Our synthetic Tyr glycopeptide library consisting of α- and β-GlcNAc- and mucin type α-GalNAc-Tyr glycopeptides were further applied to evaluate binding preferences of lectins commonly used in lectin weak affinity chromatography (LWAC) and to perform OGA and OGT enzyme assays. Antibodies directed against GlcNAc- and GalNAc-Tyr modifications are currently under development, thereby creating additional tools for enrichment and identification of Tyr glycosylation.

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(55) Mucin extended core glycopeptides to decipher lectin and antibody binding recognition events

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Mucins are densely glycosylated proteins that populate the cell-surface of epithelial tissues.[1] The extracellular tandem repeat peptide regions rich on proline, threonine and serine residues characterize the mucins. By display of *O*-glycans often organized in a multivalent fashion, the mucins and mucin like glycoproteins are involved in a plethora of cell-surface binding events.[2] By interaction with lectins the cell-surface glycans are responsible for protein cross-linking of membrane-bound receptor complexes resulting in downstream signaling events. Through the mucus layer the mucin glycans contribute to the innate immune system by providing a protecting barrier against invading pathogens. Due to the diversity and structural complexity of glycans and glycoproteins, it is a major challenge to identify and map the functions of these molecules and their interacting binding partners. Chemical synthesis of well-defined glycan and glycopeptide probes thus makes systematic binding studies feasible.

In recent years we have developed efficient synthesis strategies to construct over 200 different mucin *O*-glycopeptides modified with short tumor-associated glycan structures up to octasaccharide elongated mucin core structures.[3-5] This is one of the most complex glycopeptide libraries ever made by total synthesis and was further modified by enzymatic 2,3- and 2,6-sialylation (Neu5Ac and Neu5Gc). The synthetic glycopeptides have been applied in microarray analysis to evaluate binding epitopes of antibodies directed against tumor-associated mucin glycopeptide antigens.[5-7] Microarray analysis of lectins that are involved in tumor progression was further preformed. Analysis of virus and bacterial lectin recognition is currently in progress. Our recent findings will here be described in more detail.

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(56) L-fucose metabolism in Campylobacter jejuni

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Campylobacter jejuni is a gastrointestinal pathogen once considered asaccharolytic (1), but now known to metabolize fucose (2,3). Strains with the *fuc* locus encode enzymes for fucose uptake and metabolism and show a competitive colonization advantage in the piglet disease model (2). *C. jejuni* NCTC11168 shows reduced biofilm formation in the presence of fucose while its fucose permease mutant (*fucP*) shows no changes. However, both wildtype and the *fucP* mutant are capable of chemotaxis towards fucose. *C. jejuni* 81-176 naturally lacks the *fuc* locus and does not metabolize or swim toward fucose. However, transfer of the NCTC11168 locus into 81-176 enables the strain to metabolize fucose and show a chemotactic response toward this carbohydrate. Mutagenesis studies have linked the putative dehydrogenase Cj0485 with coordinating fucose metabolism and chemotaxis. Overall, our results suggest that fucose chemotaxis is coupled to possession of the *fuc* locus, but downstream signals, only in *fuc*+ strains, are involved in coordinating fucose availability with biofilm development.

Interestingly, the enzymes encoded by the *C. jejuni* fucose utilization locus are similar to those described for the plant pathogen *Xanthomonas campestris* (5). However, bioinformatic analyses suggests there may be minor differences in predicted functions, particularly in the involvement of a DapA aldolase (putative function of Cj0481) in the *C. jejuni* pathway (6). We are further characterizing the proteins in crystallography studies and enzymatic assays to determine their metabolic roles and are particularly interested in Cj0485 due to its dual function in both metabolism and chemotaxis. Additionally, we are interested in comparing how strains that can and cannot use fucose are able to use other carbon sources. Biolog analyses show differential utilization profiles between wildtype *C. jejuni* 11168 and the fucose regulator (*fucR*) and *fucP* mutants, suggesting that utilization of other carbon sources is linked to fucose metabolism. Furthermore, similar differences are observed when comparing carbon sources used by wildtype 81-176 to those used by 81-176 with the added fucose locus. Overall, these studies enable us to characterize a unique fucose metabolic pathway and help to better understand the importance of fucose and other carbon sources for pathogen colonization and persistence in the gut.

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(57) Fungal cell wall glucan metabolism by Bacteroides in the human gut

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Glycans are the major nutrient source for the bacteria of the human gut. The majority of these are dietary plant and animal glycan, and host carbohydrates such as mucins. Additionally, the microbes themselves produce a wide variety of cell wall and secreted polysaccharides which might also be accessible by some gut bacteria. Previously, we showed that some species of *Bacteroides*, including *Bacteroides thetaiotaomicron*, are able to degrade the outer α-mannan layer of the fungal cell wall from a number of species including *Saccharomyces cerevisiae* and *Candida albicans*, which is a commensal member of the microbiota in many healthy people, but can cause severe disease and mortality in immunocompromised individuals. We describe here a distinct polysaccharide utilisation locus (PUL) which is responsible for degrading the β-1,6 glucan component of the fungal cell wall, which forms crosslinks between the underlying skeletal β-1,3 glucan layer and the mannoproteins of the outer layer. The ability to degrade these relatively short crosslinks could aid access to the large α-mannan fibrils. This PUL contains a GH30 endo- β-1,6-glucanase, a SusCD-like TonB dependent transporter complex, a surface glycan binding protein and a GH3 exo-glucosidase. Biochemical characterisation of these enzymes and binding proteins together with a crystal structure of the GH30 in complex with theinhibitor gluco-β-1,6-deoxynojirimycin, aids our understanding of how *Bacteroides thetaiotaomicron* is able to degrade the complex glycans of the fungal cell wall.

(58) Unravelling the determinants of resistant starch utilization by human gut microorganisms

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The gut microbiome can have numerous effects throughout the body. These effects are largely mediated through small molecules, such as the short chain fatty acids (SCFA) that are the end product of carbohydrate fermentation in the gut. Of the SCFA, butyrate is regarded as the most beneficial, serving as the primary energy source of colonocytes and exhibiting anti-inflammatory, anti-diabetic and anti-tumorigenic effects. Prebiotic intake can lead to increased butyrate levels and in particular the dietary fiber resistant starch is known to be highly butyrogenic. However, little is known about how resistant starch, which cannot be broken down by human enzymes, is processed by the gut microbiota.

Eubacterium rectale is an important butyrate producing organism in the gut. It is able to grow on regular starch, but not resistant starch. In contrast *Ruminococcus bromii* is known to be able to grow on resistant starch, but is not a butyrate producing organism. To unravel the factors that allow resistant starch utilization, a comparison between these two organisms has been undertaken. *E. rectale* uses a simple system for degrading starch, relying on a single extracellular enzyme and a suite of transporters to effect growth on starch. Structural and binding analysis of the carbohydrate binding modules of this protein demonstrate that they are unable to target resistant starch, providing one reason why this organism cannot utilize that substrate. In contrast *R. bromii* utilizes a comparatively complex enzyme system that forms a cellulosome-like multi-enzyme complex we call the amylosome. This system allows the combining of enzymatic activities into a single molecule for the efficient attack of its substrate. As part of this system, *R. bromii* encodes a suite of pullulanases that can target the branch points in starch, a potential weak point in the otherwise recalcitrant resistant starch. Biochemical and structural analysis of these debranching enzymes has revealed subtly different specificities that may allow the targeting of a variety of starches, including resistant starch. Together these results allow us to begin to form a model for how resistant starch is used by the gut microbiota.

(60) Pivotal alpha mannosidase generates specificity for N-glycans through requirement for GlcNac at +2 subsite.

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In the human gut microbiota *Bacteroides thetaiotaomicron* adopts a glycan generalist strategy to utilise the vast array of indigestible dietary and host polysaccharides as a carbon source. While many of the Polysaccharide Utilisation Loci (PULs) which target dietary glycans have been characterised, less is understood about N-glycan utilisation. High mannose N-glycans are abundant in the gut from host proteins and glycosylated fungal proteins and also provide the core pentasaccharide for complex and hybrid N-glycans. *B. thetaiotaomicron* has a dedicated HMNG PUL consisting of an endo-β-N-acetylglucosaminidase, glycoside hydrolase (GH) family 18, which removes the N-glycan from the protein at the cell surface. The glycan is transported in to the periplasm, where a number of α-mannosidases, from family GH92, further digest the glycan from Man9GlcNAc to leave a trisaccharide of Man-α1,6-Man-β1,4-GlcNac. The HMNG PUL does not encode the enzymes necessary to degrade this trisaccharide, a further GH92 and a GH130 are encoded in a distinct operon from the HMNG PUL which is expressed at a high basal level. Here we describe the

biochemical and structural characterisation of an α-1,6 mannosidase from this operon which requires GlcNac in the +2 subsite of the active site. The requirement of the +2 subsite to be occupied is unique for GH92 enzymes and suggests this enzyme has a specific role in processing of the Man-α1,6-Man-β1,4-GlcNac product of the HMNG PUL. A genetic knockout of the enzyme in *B. thetaiotaomicron* confirms its role in the degradation of HMNG, and likely in processing the core of complex and hybrid N-glycans from other sources. The need for these two enzymes to work downstream of multiple N-glycan PULs could explain why they are highly expressed throughout all growth conditions tested.

(61) Degradation of complex N-glycans by gut Bacteroides species

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Humans and their gut microbiota have a symbiotic relationship. Humans provide the gut microbiota with sources of energy in the form of glycans from ingested foodstuffs recalcitrant to humans and mucosal glycans produced by the host. In return, an estimated 10 % of calories from a human diet come from the fermentation of polysaccharides to short chain fatty acids by the gut microbiota. Interactions between humans and the gut microbiota have also been linked to many aspects of human health and disease. Bacteroidetes are one of the two main phyla found in the human gut and can use a wide variety of dietary and host glycans as their source of nutrients. The genes required for the catabolism of a particular polysaccharide are typically grouped together on the genome in 'polysaccharide utilisation loci' (PULs) and the number of PULs per substrate can be directly related to its complexity.[DB1] N-glycans are found decorating predominantly eukaryotic proteins so gut bacteria will see N-glycans from other gut inhabitants (such as fungi), host proteins and plant and meat dietary sources. Growth of various Bacteroides spp. on host glycans has allowed the identification of a number of putative N-glycan targeting PULs. Here we show that various Bacteroides spp can access N-glycans as a nutrient source and have explored the mechanisms through which the different species target various forms of complex N-glycans. In doing so, we have characterised a number of enzymes with new specificities and activity. From Bacteroides thetaiotaomicron, these include a GH18 to remove entire N-glycan structures, a GH20 that can tackle a number of different linkages and an unclassified GH enzyme capable of removing LacNAc structures from complex N-glycans. From Bacteroides massiliensis, we have characterised a PNGase and a GH92 which show specificity for plant-type complex N-glycans by accommodating xylose and fucose decorations. Overall these data provide significant insight into how different N-glycans are accessed as a nutrient source by the microbiota.

(62) An integrative strategy to decipher glycan recognition in the human gut microbiome

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The human gut houses a dense microbial community - the microbiome. Members of this diverse microbial population exhibit a broad capacity to metabolise dietary and host-derived glycans[1]. This is essential for maintaining human health including nutritional balance, intestinal homeostasis and immune system modulation. Other activities variously promote human disease.

Gut microbial strains isolated exhibit a high number of gene clusters termed polysaccharide utilisation loci (PULs) that orchestrate recognition and degradation of specific glycoconjugates, allowing the bacteria to cope with nutrient fluctuations[2]. Intensive research on PULs has shed light into their complex architecture, which includes modular carbohydrate-active enzymes with associated carbohydrate-binding modules (CBMs), starch-utilisation system (Sus)-like proteins and cell surface glycan-binding proteins (SGBPs). The number of PULs identified by genomic and transcriptomic analyses and bioinformatics is growing fast and many of these await assignment of glycan-binding functions.

In this communication, we will present our integrative strategy to interrogate, in a high-throughput manner, specific microbiome-human glycan-recognition systems. Currently, we are focusing on PULs proteins from two representative strains of the gut microbiota, *Bacteroides thetaiotaomicron* and *Bacteroides ovatus*, which have adapted to mammalian- or dietary-derived glycans, respectively[1]. Our strategy includes: bioinformatics, sequence analysis and production of a recombinant-protein library (around 100 different protein domains at present); development of glycan microarrays comprising a diverse range of mammalian- fungal- or plant-derived glycan probes, for screening analysis of glycan-binding[3,4]; and structural characterization of newly identified proteins and protein-oligosaccharide interactions using X-ray crystallography[5,6]. This combined strategy can be further applied to other microbial strains and will contribute to a better understanding of the functions of PULs as well as to a broad understanding of the human microbiome metabolic capabilities.

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(63) Bacteroides thetaiotaomicron requires rhamnose release to grow with Gum Arabic

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Bacteroides cellulosilitycus (Baccell) is a human host able to degrade multiple complex polysaccharides as a carbon source. The glycan component of Type II arabinogalactan proteins (AGPs) has a wide range of chemical structures that are plant specific. Larch wood AGPs have been widely studied and the chemical structure has been clarified. The structures of the glycans in AGPs from several plant species, including wheat, carrot, coffee and Arabidopsis leaves, have also been elucidated. They all contain a beta-1,3-D-galactan backbone with beta-1,6-D-galactan side chains. The length of these galactan side chains and their decoration with additional sugars varies between plant species. In the case of Gum Arabic (GA), multiple studies have reported different side chain structures, although the monosaccharide composition is frequently reported to comprise galactose, arabinose, GlcA, Me-GlcA, and rhamnose. Baccell has the enzymatic machinery necessary to degrade natural AGPs like GA or wheat completely. We described the PULs regulated in the AGP degradation in Baccell and the proteins implicated in this degradation. In this order, we provide an enzymatic model about how these complex substrates are degraded by this commensal. This is the first time that one *Bacteroides* strain can degrade natural AGPs like wheat, carrot, GA or extensin, another hydroxyprolin rich glycoproprein. In the case of *Bacteroides thetaiotaomicron* (BT), another commensal of the human gut, it has been described that can use LAG as a carbon source but cannot do it with physiological AGPs like wheat, carrot or GA (1). However, in the case of GA, this problem was solved when the polysaccharide is treated in vitro with an enzyme releasing rhamnose. The localization of this protein in BT is wrong maybe forced by the evolution in the human gut. As a consequence, BT needs to degrade AGPs from the human diet with the help of other *Bacteroides* like Baccell in a sharing manner.

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(64) Insight into N-glycan breakdown by the gut microbiota

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The colonic microbiota plays a significant role in the maintenance of human health. Survival in this competitive niche depends on the ability of specific microorganisms to utilise complex glycans as nutrients as these molecules cannot be accessed by the host and thus escape digestion in the upper GI tract. While specific members of the normal gut microbiota are known to utilise plant derived glycans and host O-glycans, it is currently unclear if complex N-glycans are a nutrient source for the microbiota and if so how they are accessed. N-glycans are found in the gut on glycoproteins from both host and dietary sources and encompass a range of different structures. Here we show that *Bacteroides thetaiotaomicron* (*Bt*), a prominent member of the colonic ecosystem, can degrade a range of complex N-glycan structures and describe the biochemical and genetic characterisation of a seven enzyme polysaccharide utilisation locus (PUL) from *Bt* involved in complex N-glycan deconstruction. These include the only sialidase (GH33) in *B. thetaiotaomicron*, two GH2s and three GH20s that target a number of different complex N-glycan linkages. The data provide insight into N-glycan metabolism by the microbiota and suggest that breakdown of specific N-glycan structures by *Bt* is a cooperative process involving multiple PULs.

(65) Analysis Human Microbiome Reveals a New Glycoside Hydrolase Family, Which Lacks the Canonical Catalytic Apparatus

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The human distal gut is a highly competitive microenvironment where limited carbon sources, in the form of complex glycans, are competed for by a vast number of microorganisms. These factors combine to place an evolutionary pressure on the microbial community in the gut, whose combined genome is 100 times greater than that of their human hosts. This makes this microbial community a huge resource for discovering novel Cazymes. By using a complex glycan, in this case acai gum Arabic (GA), for which a full 'degradome' is not known we can discover new enzymes and new activities.

This approach, using *Bacteroides thetaiotaomicron* (*Bt*) as the model organism, led to the discovery of a new glycoside hydrolase (GH) family. This new family targets α 1,4 linked L-rhamnose linked to glucuronic acid. The enzyme is the first GH family to have a type-A seven bladed propeller. The family also displays a form of circular permutation with the active site being located on the opposite side of the propeller compared to all other GH beta propeller folds characterised to date. This active location defies common logic

with it being located at a site of least conversion; with most conservation being observed on the side of the propeller opposite to the active site.

Furthermore the enzyme performs catalysis lacking the canonical catalytic residues. For GHs these are usually a pair of carboxylates. NMR analysis showed that BT3686 performed catalysis causing retention of the anomeric configuration inferring a classic double displacement mechanism. Structural, biochemical and bioinformatics analysis of BT3686 and 5 homologs from related *bacteroides* strains identified a histidine as the critical catalytic residue. Structural analysis infers that this histidine may proceed through an epoxide intermediate with substrate assisted catalysis rather than the more standard double displacement mechanism. Finally, and critically, in homologs that lacked the rhamnosidase activity, and the critical histidine, we were able to engineer rhamnosidase activity into these apparently inactive enzymes. This work highlights the enormous potential of the human microbiomes vast genetic diversity as well as opening up new avenues in protein design when considering a beta propeller scaffold.

(66) Mucin-type O-glycans are essential for homeostasis between host and microbiota in the colon

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Background. Core 1- and core 3-derived mucin-type *O*-glycans are primary components of the colonic mucus layer. Reduced mucus thickness, impaired *O*-glycosylation, and aberrant activation of inflammasome-dependent inflammatory pathways are observed in human ulcerative colitis (UC), but whether these phenotypes are linked is unclear. **Methods.** To address this, we analyzed the intestinal expression of C1GalT1 and C3GnT, which control biosynthesis of core 1- or 3-derived *O*-glycans in mice. We further compared mucus barrier function, colitis susceptibility, inflammasome activation, and microbial communities between WT mice, and mice lacking core 3 (*C3GnT*$^{-/-}$), intestinal epithelial core 1 (IEC *C1galt1*$^{-/-}$), and/or both types of *O*-glycans (DKO). **Results.** We found *C1galt1* expression was highly expressed throughout the WT small intestine and colon, while *C3GnT* expression was mainly restricted to the proximal colon and cecum. Consistent with this, IEC *C1galt1*$^{-/-}$ mice developed spontaneous colitis mainly in the distal colon, whereas colitis in DKO mice occurred in both the distal and proximal colon. Colitis induction was directly correlated with mucus barrier dysfunction, which was significantly impaired in DKO mice. The colitis and mucus phenotypes were microbiota-dependent as colitis was reduced and mucus layer restored in antibiotic-treated DKO mice. In addition, we identified increased activation of epithelial caspase 1 (casp1)-dependent inflammasomes in DKO vs. WT mice, and DKO mice lacking inflammasomes showed significantly reduced disease vs. DKO littermates despite absence of the mucus barrier. Illumina MiSeq of the 16S rRNA gene amplified from luminal content showed consistently altered community composition in DKO vs. WT co-housed littermates; however, known colitic bacteria belonging to the Bacteroidetes phylum were highly represented in both strains (20 – 30% of total OTUs at genus level). FISH analysis on colon sections confirmed strong Bacteroidetes representation, but their spatial distribution changed from luminal in WT mice to mucosa-associated in DKO. **Conclusions.** These studies demonstrate that mucin-type *O*-glycosylation plays a key role in intestinal homeostasis by promoting integrity of the mucus layer in the presence of potentially colitic microbiota, and by regulating the activity of epithelial casp1 activation to promote its homeostatic functions while limiting its pathogenic roles.

(67) The deleterious effect of AB5 toxins on Campylobacter jejuni strains that mimic GM1 ganglioside: a means of bacterial warfare.

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Campylobacter jejuni is a leading cause of gastroenteritis worldwide and can lead to serious post infectious sequelae due to its ability to mimic human gangliosides through its lipooligosaccharide (LOS) structures. This can lead to Guillain-Barré Syndrome due to production of self-reactive antibodies that target nerve cells. The mimicry also allows both cholera toxin (CT) of *Vibrio cholerae* and heat-labile enterotoxin (LT) of *Escherichia coli* to bind *C. jejuni* strains that display the toxins' GM1 ganglioside receptor. Our preliminary results showed that this interaction has the ability to reduce *C. jejuni* growth. The binding and clearing effects of these toxins were examined via transmission and scanning electron microscopy. Fluorescent microscopy using SYTOX®Greennucleic acid stain suggests that the clearance effect is related to membrane damage and likely cell death. Interestingly, *C. jejuni* 11168 is capable of phase-varying the β-1,3 galactosyltransferase encoded by the wlaN gene, modulating its ability to mimic the ganglioside and bind the toxins. When LOS is isolated from *C. jejuni* 11168 after exposure to CT or LT and compared to wildtype LOS, there is a low molecular weight band lost on silver stained SDS-PAGE gels that corresponds to a significant drop in CT binding by Far Western blot analyses. These observations paralleled a change in the wlaN sequence to favour inactivation of the galactosyltransferase gene, further supporting that these toxins are acting as selective pressures to alter ganglioside mimicry in *C. jejuni*. However, these toxins were unable to clear growth of *E. coli* strains that were

engineered to display the same GM1 mimic, suggesting that the effect is specific to organisms that naturally produce the target. The bactericidal activity of AB5 toxins secreted by organisms competing for the same intestinal niche as *C. jejuni* suggests that there may be alternate functions for these toxins in bacterial warfare in addition to their well-characterized roles in causing severe human diarrheal disease.

(68) Discovery and Implication of a Unique Extracellular Polysaccharide in Members of the Pathogenic Bacillus that can Co-form with Spores

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The spore causing anthrax disease was studied in details for years. As a bioweapon, the tough dormant spore is stable and successfully survived throughout harsh environments for long period of time. While all attention was given to the naked spore, an unusual linear polysaccharide, named pzX, was recently found to be released during spore formation in all three highly phlyogenetically conserved pathogenic *Bacillus* strains: *B. anthracis*, *B. cereus*,and *B. thuringiensis*. pzX consists of a rare sugar residue XylNAc that was never found in any glycan structure. Biochemical and genetic data provide evidence that the six-gene operon, XNAC, is exclusively present in pathogenic *Bacillus cereus* sensu lato group, and is responsible for pzX formation.

We suggest that the existence of pzX must be considered in order to fully understand how *Bacilli* survive, adapt, and are recognized by diverse types of environments and hosts. For instance, when pzX co-dries with spores it increases their adherence to surfaces. Other distinct properties involving pzX-spore biology will be discussed.

The control for pzX formation is tightly regulated; for example, high glucose that appears to suppress spore formation also prevents pzX formation. However, below a threshold, glucose increases pzX synthesis. We will present other data involved in the regulation of pzX.

While pzX is unique to *Bacillus*, we propose that extracellular polysaccharide co-formed with spores is a universal phenomenon. This phenomenon is likely shared by other spore-forming organisms including the notorious pathogen *clostridium*.

(69) Identification of influenza A virus receptors found in natural tissue using shotgun glycomics approach

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Influenza A virus (IAV) hemagglutinin (HA) proteins facilitate viral entry by binding to cell surface receptors terminating in sialic acid, the linkage of which is correlated with host species specificity. Avian HAs recognize **α**2,3 linked terminal sialic acid and human and swine viruses exhibit a preference for **α**2,6 linked terminal sialic acid. Other structural determinants beyond the terminal sialic acid linkage that may be important for receptor binding of IAV have not been studied in great detail. The goal of this work is to study the glycan structures found in natural tissues, and determine what other features, if any, have implications for HA binding and species specificity. We used novel shotgun glycomics technology to identify the natural endogenous glycans bound by IAV HA. N-glycans, O-glycans, and glycolipid-derived glycans were isolated from the tissue, fluorescently tagged, and separated using multi-dimensional HPLC. Pig lung shotgun glycan microarrays were generated from the purified glycan species. A diverse panel of influenza viruses representing human, avian, and swine hosts as well as a variety of HA subtypes and dates and locations of isolation was used to interrogate the microarrays. Virus binding allowed for prioritization of structural characterization with the highest binding species being subjected to metadata- assisted glycan sequencing. It was revealed that all of the panel viruses only bound to sialylated N-glycans, not O-glycans or glycolipid-derived glycans. In cases of limited recognition of structures within the 2,3 or 2,6 linkage group, other structural determinants in addition to the sialic acid, such as core fucosylation, influence virus binding. Notably, many of the structures characterized as IAV receptors were not present on the available synthetic arrays, highlighting the importance of continued study with natural/endogenous glycan structures. Through this work, we have characterized a set of natural glycans as potential endogenous receptors of IAV in the swine host and discovered structural modifications that influence IAV HA binding. The shotgun glycomics approach will allow for the examination of the glycans of other natural biologically-relevant tissues, enabling the study of the molecular determinants of cross-species host receptor adaptation that might be a precursor to cross-species transmission of influenza viruses.

(70) The glycan receptors of Helicobacter pylori: decoding the pathways underlying gastric glycophenotype modulation

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The gastrointestinal tract is covered by a complex extracellular mucus layer that protects the gastric epithelium from external aggressions such as chemical agents, microorganisms and shear stress. Although this mucus barrier confers protection against certain pathogens, it may also provide a niche for microbial binding. *Helicobacter pylori* exploits the host glycoconjugates present in the gastric mucus layer and lining the surface epithelium of the gastric mucosa to colonize the stomach. Infection can persist for decades promoting chronic inflammation, and in a subset of individuals lesions can silently progress to cancer. The secreted MUC5AC mucin is the main component of the gastric mucus layer, and *H. pylori* BabA-mediated binding to MUC5AC confers increased risk for overt disease. We have shown that FUT2 determines the *O*-glycosylation pattern of Muc5ac, with Fut2 knock-out leading to a marked decrease in α1,2-fucosylated structures and increased expression of the terminal type 1 glycan structure Lewis^a. Importantly, for the first time, we structurally validated the expression of Lewis^a in murine gastric mucosa (1). We further demonstrated that loss of mucin FUT2-mediated fucosylation impairs gastric mucosal binding of *H. pylori* BabA adhesin, which is a recognized feature of pathogenicity. Upon *H. pylori* infection,concomitantly with tissue inflammation, there is a remodeling of the gastric glycophenotype. We showed that increased expression of sialyl-Lewis$^{a/x}$ antigens is due to transcriptional up-regulation of the *B3GNT5*, *B3GALT5*, and *FUT3* genes. In addition, we observed that *H. pylori* infected individuals present a marked gastric local pro-inflammatory signature with significantly higher TNF-α levels and demonstrated that TNF-induced activation of the NF-kappaB pathway results in B3GNT5 up-regulation (2). Furthermore, we showed that this gastric glycosylation shift, characterized by increased sialylation patterns, favors SabA-mediated *H. pylori* attachment to human inflamed gastric mucosa. Our data provides clinically relevant insights into the regulatory mechanisms underlying *H. pylori* modulation of host glycosylation machinery, and phenotypic alterations crucial for life-long infection and gastric disease.

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(71) ADP-ribosylation in the innate immune response

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ADP-ribosylation is a post-translational modification (PTM) important for DNA repair and inflammatory signaling. While inhibition of poly(ADP-ribose) polymerases (PARPs) – the enzymes responsible for ADP-ribosylation – is known to protect animal models from sepsis, the molecular mechanisms behind this response are unknown. In order to investigate the role of this PTM in the innate immune response, we have characterized ADP-ribosylation in macrophages, both before and after activation by lipopolysaccharide (LPS) – a molecule found on Gram-negative bacteria. The macrophage ADP-ribosylated proteome changes in response to LPS treatment, which we have shown by both western blot and mass spectrometry, for both human and mouse cells – this dataset represents the first draft of the macrophage PARylated proteome. The mass spectrometry results were acquired through application of our recently described method wherein ADP-ribose is cleaved down to its phosphoribose attachment site and then phosphoenriched – a pipeline, which delivers both the phosphoproteome and the ADP-ribosylated proteome. Furthermore, we have shown by both fluorescent microscopy and Cytometric Bead Array technology that NFkB and the cytokines associated with its activation are altered in response to PARP inhibition, an effect, which is dramatized by macrophage activation with LPS. Finally, we have shown that TLR4 is ADP-ribosylated on its TIR domain, an otherwise unknown modification on an important regulatory domain for all TLR signaling. The presence of this modification site, the importance of TLR4 signaling in sepsis pathogenesis, and the protective effect of PARP inhibition against sepsis, suggests that ADP-ribosylation may be a major player in TLR4 signaling. Our dataset represents the first proteome-wide assessment of both human and mouse macrophage ADP-ribosylation, and implicates PARP biology in the early innate immune response.

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(72) Function and mechanisms of O-fucosylation of malaria parasite TSR-domain proteins

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The thrombospondin type I repeats (TSR) domains are commonly fucosylated by the protein-*O*-fucosyltransferase 2 (PoFUT2) and this modification is required for optimal folding and secretion of TSR-containing proteins. The human malaria parasite *Plasmodium falciparum* expresses proteins containing TSR domains, such as circumsporozoite and TRAP-related protein (CTRP) and the circumsporozoite protein (CSP), that play essential roles in motility and subsequently mosquito and human host cell invasion, during the parasite's sexual and sporogonic life stages. A PoFUT2 homolog is conserved and expressed by *P. falciparum*, and GDP-fucose, the substrate donor of *O*-fucosylation reactions, is actively synthesized and incorporated by the parasite. Moreover, the *O*-fucosylation machinery has been detected in salivary gland sporozoites by proteomic analyses, pointing to the conservation of a PoFUT2 mediated *O*-fucosylation mechanism in *P. falciparum*. We have generated, by a double crossover recombination strategy, a null mutant in *Pf*PoFUT2 to characterize its phenotype, to confirm the involvement of this gene in TSR-domain *O*-fucosylation and to assess the biological significance of TSR *O*-fucosylation modification in *P. falciparum* development. The *Pf*PoFUT2 genetic disruption did not affect growth of the asexual stages of the parasite, exhibiting similar growth kinetics to the parental NF54 'wild type' strain in culture. Since CTRP and CSP, two critical TSR-containing proteins, are expressed during the transmission stages of the parasite, we analyzed the developmental phenotype in its mosquito vector, *Anopheles gambiae*. The number and size of oocysts present on the mosquito midgut did not differ between the null mutant and the wild type strain; showing that the lack of *O*-fucosylation does not seem to affect parasite invasion of the midgut epithelium. Furthermore, both mutant and wild type parasite strains show comparable numbers of sporozoites in the salivary glands. We are currently quantifying the effect of the *Pf*PoFUT2 disruption on sporozoite motility and human hepatocyte invasion.

(73) Early remodeling of the hepatocyte glycocalyx during hepatitis C virus infection: toward the settling of viral persistence and chronicity?

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Over 30% of hepatocellular carcinoma cases worldwide are a consequence of a chronic infection with the hepatitis C virus (HCV). However, unlike other oncogenic viruses, HCV does not integrate its genome into that of hepatocytes, its host cells. It is therefore unlikely that direct genomic disturbance would prevail at the onset of HCV-induced hepatocarcinogenesis. To reach hepatocytes, HCV virions cross the space of Disse while leaving the blood stream through the fenestrae of sinusoid capillaries. Therein, they encounter the hepatic extracellular matrix (ECM). Chronic hepatitis C causes the accumulation of scarring ECM in the space of Disse, leading to liver fibrosis and cirrhosis. These symptoms underline the extreme reshaping of hepatic ECM during HCV infection.

Our study focuses on the hepatocyte glycocalyx, a specific ECM area defined as the layer of glycoproteins and proteoglycans anchored in the plasma membrane. We aim at dissecting the cascade of events whereby HCV infection reshuffles this area, with a particular interest in heparans sulfates proteoglycans (HSPG). Our ultimate goal is to understand how this cascade might translate into early events of carcinogenesis.

Within days after HCV infection, we observed a downregulation of syndecan-1, a particular transmembrane HSPG described as an HCV attachment factor. We uncovered major virus-induced perturbations of the upstream enzymatic cascade involved in HSPG biosynthesis. These dysfunctions occurred on a similar timescale as syndecan-1 downregulation, and were concomitant to a steep decline in the intracellular level of xylose, the first and key sugar grafted onto the HSPG core. Furthermore, we addressed the question of a viral-induced shedding of syndecan-1, a process that would release the soluble ectodomain of syndecan-1 in the space of Disse at abnormal levels, thereby contributing to early alterations of the hepatic ECM. These features would likely be translated into alterations of the glycosylation profiles of HPSG of hepatocytes, a point that we currently investigate.

These data underscore a major reshuffle of the hepatocyte glycocalyx, paralleling HCV replication. We are currently investigating the relation between this phenomenon and establishment of chronicity, as this could give novel insights into the oncogenic mechanisms linked to HCV infection.

(74) Chlorella viruses: antigenic variants act as tools to correlate gene-to function of protein A064R, an apparent multifunctional glycosyltransferase

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Nucleocytoplasmic large DNA viruses (NCLDV), such as *Paramecium bursaria* chlorella virus (PBCV-1), are a group of viruses characterized by a large particle size and very large genomes. These viruses encode proteins involved in functions not normally found in viruses, including at least part of a host-independent autonomous glycosylation pathway.

PBCV-1 encodes at least six putative glycosyltransferases (1): A064R (638 amino acids), A075L (280 aa), A111/114R (860 aa), A219/222/226R (677 aa), A473L (517 aa), and A546L (396 aa). We believe that the virus PBCV-1 (2) encodes the proteins responsible for the assembly of some or all of the 9 (sometimes 10) monosaccharides that comprise the major capsid protein glycan. If true, how do six glycosyltransferases assemble such a large N-linked oligosaccharide? Some of the six glycosyltransferase genes encode large proteins, suggesting that they may be multifunctional, making this restricted repertoire of enzymes sufficient to synthetize the N-glycans.

Regarding the multifunctional character of some of the putative PBCV-1 transferases, one enzyme of interest is A064R: it contains three domains of ca. 200 aa each. Domain 1 has the typical DXD sequon that coordinates the phosphate of a UDP-nucleotide sugar along with a divalent cation (1). Domain 3 has a weak match with methyltransferases. Domain 2 only matches proteins of unknown function, but genetic analysis suggests that it also has a glycosyltransferase function.

Interest in the A064R gene stems from the fact that 18 of 21 mutants map to this gene. These mutants, called antigenic variants, are divided into six antigenic classes based on their differential reaction to six different polyclonal antibodies. Mutants in five of the six antigenic classes have been genetically mapped to the a064r gene.

These viruses have no mutations in the gene that encodes the major capsid protein, but all of the antigenic mutants exhibit truncated glycan structures that depend on the location of the genetic mutation (1). Different N-glycan structures are produced, even though only one of the A064R domains resembles a glycosyltransferase.

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(75) Design of a Influenza A virus-glycan interaction map (glycointeractome)

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Influenza A virus is the most common influenza type and can cause serious disease in humans and animals. Human infections with the pandemic H1N1 ('swine flu'), the highly pathogenic avian H5N1 ('bird flu'), and the more recent avian H7N9 have caused widespread public concern. Especially the avian H5N1 strain has shown high mortality rate in human but has not yet adopted an efficient human-to-human transmission that potentially could lead to the next deadly pandemic. The growing resistance to the commonly used anti-influenza drug oseltamivir carboxylate (Tamiflu®) emphasises the need for the development of novel antivirals. To accomplish this goal it is essential to gain structural knowledge of the mechanism by which influenza viruses attach and enter the host cell.

We have developed a unique protocol using Nuclear magnetic resonance (NMR) spectroscopy that allows us to directly investigate the interaction of influenza virus particles with host cell carbohydrate receptors (glycans) at an atomic level. In combination with cell-based assays our approach has successfully been used to characterize the interactions of the viral surface glycoprotein hemagglutinin with cell surface glycans.

A virus-glycan interaction map (glycointeractome) will be presented that not only advances our understanding of virus specificity and host cell tropism but is also of crucial importance to guide structure-assisted design of the next generation of broad-spectrum anti-influenza drugs.

(76) The Structure of the UDP-Glc/GlcNAc 4-Epimerase from the Human Pathogen Campylobacter jejuni

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Worldwide, the food-born pathogen *Campylobacter jejuni* is the leading bacterial source of human gastroenteritis. *C. jejuni* produces a large number of atypical cell-surface carbohydrates that facilitate infections. A major component of these oligo- and polysaccharides is the sugar *N*-acetylgalactosamine (GalNAc). The sole source of this critical sugar is derived by the epimerization of UDP-*N*-acetylglucosamine (GlcNAc) catalyzed by the enzyme UDP-GlcNAc 4-epimerase (GNE). This enzyme is unique among known bacterial epimerases in that it also catalyzes the equivalent reaction with the non-*N*-acetylated sugars. Understanding how *Cj*GNE catalyzes these various interconversions is critical to designing novel inhibitors of this enzyme. Here, to further the mechanistic understanding we present a 2.0Å crystal structure of *Cj*GNE with its NAD^+ co-factor bound. Based on novel features found in the structure we perform a variety of biochemical studies to probe the mechanism and compare these results to the only other structurally characterized bi-functional epimerase, the human homolog GalE. We further show that ebselen, previously identified for inhibition of *Hs*GalE, is active against *Cj*GNE, suggesting a route for antibiotic development.

(77) Protein glycosylation in Campylobacter jejuni: Deciphering the role of the N-glycan on the CmeABC efflux pump

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Campylobacter jejuni is the most common bacterial cause of gastroenteritis in humans. *C. jejuni* was the first bacterium demonstrated to possess a general N-linked protein glycosylation (*pgl*) pathway that adds a heptasaccharide consisting of GalNAc-GalNAc-(Glc)GalNAc-GalNAc-GalNAc-diNAcBac to the sequon D/E-X1-N-X2-S/T (where X1/X2 are not proline) of more than 60 periplasmic and membrane proteins. We previously demonstrated that Pgl pathway glycans are also released into the periplasm as free oligosaccharides (fOS) by the oligosaccharyltransferase (PglB). FOS and N-glycan structures are conserved among thermophilic *Campylobacters* but are structurally diverse in non-thermophilic strains. While fOS production in *C. jejuni* has been shown to respond to the osmotic environment of the cell, the exact role of the N-glycan on proteins remains unknown.

We show that N-glycosylation is required for the function of the major CmeABC efflux pump that supports bacterial survival in the presence of antibiotics and bile salts. A *cmeA* mutant is highly susceptible to erythromycin, ciprofloxacin and bile salts when compared to the wild-type strain. Complementation of the *cmeA* mutant with wild-type *cmeA* restores the wild-type phenotype whereas expression of a *cmeA* allele with point mutations in both glycosylation sites was comparable to the *cmeA* mutant. Moreover, we show that loss of CmeA glycosylation led to reduced chicken colonization levels similar to the *cmeA* knock-out strain, whereas the mutant complemented with the wild-type *cmeA* allele restored colonization. In addition, reconstitution of the *C. jejuni* CmeABC in *E. coli* in the presence or absence of the campylobacter protein glycosylation pathway revealed a 4-fold increase in the MIC towards erythromycin compared to cells lacking the *pgl* pathway.

Molecular dynamics simulations comparing the N-glycan in more detail revealed that the protein backbones of the glycosylated and non-glycosylated CmeA did not exhibit large variations from one another. Further studies demonstrated that although the addition of the N-glycan partially protects CmeA from proteolytic degradation *in vitro*, the N-glycan does not seem to influence CmeABC complex formation and stability *in vivo*. Therfore we conclude that N-glycosylation of CmeABC most likely directly influences the function of the CmeABC complex rather than its stability.

(78) Structure-activity relationship (SAR) study on the role of L-fucose in cholera toxin binding to intestinal epithelial cells

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Cholera toxin (CT) utilizes the thick glycan coating on the plasma membrane of the intestinal epithelium to invade host tissue, and its retrograde transport to the cytosol results in the massive loss of fluids and electrolytes associated with severe dehydration. It is widely accepted that the monosialoganglioside GM1 is cholera toxin's sole receptor to initiate this process. However, our lab recently determined that in intestinal epithelial cell lines that express very low (almost undetectable) levels of the GM1 lipid, it is fucosylated glycan epitopes on glycoproteins that are contributing to the cell surface binding and endocytic uptake of the toxin. In this work, we have expanded our competition binding assays with L-fucose to encompass stereochemical and positional analogs, in order to decipher which parts of the sugar ring are required for inhibition of cholera toxin subunit B (CTB) binding. Furthermore, an increase in potency is observed with the use of mono- and di-fucosylated oligosaccharides as inhibitors, of which the LeY tetrasaccharide emerged as being the most potent inhibitor when tested against a blood group O cell line. Finally, non-natural fucose-containing polymers that inhibit CTB two orders of magnitude more potently than the LeY glycan are being developed. Future work entails identification of residues within the CTB binding site that are contacted by these fucosylated structures, as well as the glycoproteins that mediate toxin binding to the cell surface, in the hopes that biomimetics can be developed for the specific blocking of CT targeting to the intestinal epithelium.

(79) Characterization of the synthesis pathways of acylated dideoxyhexosamines in Campylobacter jejuni strains with lipooligosaccharide biosynthesis loci E and H

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Campylobacter jejuni *strains with lipooligosaccharide (LOS) biosynthesis loci E, H, O, P and W, share a cluster of 5 genes encoding enzymes that are proposed to synthesize dTDP activated donors of dideoxyhexosamines (ddHexN). This gene cluster is followed by two putative* N-*acyltransferase genes that vary between strains. LOS outer core structural data are available for only two strains, a class H one (ATCC 43431, Penner serotype HS:3) and a class E one (81116, Penner serotype HS:6). The ATCC 43431 LOS outer core includes 3-acetamido-3,6-dideoxy-D-glucose (Qui3NAc) while the 81116 LOS outer core was proposed to include two ddHexN residues that can be substituted with either an* N-*formyl group or an* N-*acetyl group. In order to determine the ddHexN biosynthetic pathways in these strains, we cloned and expressed genes that were identified by sequence homology to be involved in activated donor synthesis. Five genes were used in this study, namely* rmlA, rmlB, wlaRA, wlaRB *and* wlaRG. *RmlA has homology with glucose-1-phosphate thymidylyltransferases which synthesize dTDP-Glc from dTTP and glucose-1-phosphate. RmlB is homologous with dTDP-D-glucose 4,6-dehydratases and is*

proposed to convert dTDP-Glc to dTDP-4-keto-6-deoxy-D-glucose. WlaRA and WlaRB are proposed to be dTDP-4-keto-6-deoxy-D-glucose-3,4-ketoisomerases which would convert dTDP-4-keto-6-deoxy-D-glucose to distinct products. WlaRG was previously proposed to be a dTDP-3-keto-6-deoxy-hexose PLP-dependent aminotransferase based on sequence homology and gene specific knock-out. The genes encoding these five enzymes were cloned and expressed in Escherichia coli*, and then the enzymes were purified. We confirmed the functions of RmlA and RmlB by synthesizing dTDP-4-keto-6-deoxy-D-glucose starting with glucose-1-phosphate and dTTP. Using dTDP-4-keto-6-deoxy-D-glucose as substrate, WlaRG was used in coupled assays with either WlaRA or WlaRB which resulted in the production of dTDP-α-Fuc3N (dTDP-3-amino-3,6-dideoxy-D-galactose) or dTDP-α-Qui3N (dTDP-3-amino-3,6-dideoxy-D-glucose), respectively. Mass spectrometry and NMR analyses were used to confirm the identity of the products. This study shows that* C. jejuni *81116 uses these five enzymes to synthesize glucose and galactose configured 3-amino-3,6-dideoxy dTDP-sugar intermediates. These ddHexN intermediates can then be further modified by WlaRC, a sugar* N*-acetyltransferase or WlaRD, a sugar* N*-formyltransferase.*

(80) Conformation of the 216-loop of human parainfluenza type 1 hemagglutinin-neuraminidase determines inhibitor selectivity

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Human parainfluenza virus type-1 (hPIV-1) is one of the major etiological agents responsible for respiratory tract infections in young children, along with the elderly and immunocompromised. To date, there is neither vaccine nor clinically available treatments to prevent or treat hPIV infections. The hemagglutinin-neuraminidase (HN) is a key hPIV surface glycoprotein. It binds to, as well as cleaves sialic acid-containing receptors on host-cells and promotes fusion of the virus envelope with the cell membrane. Due to its involvement at multiple points in the infection cycle, HN is an ideal target for drug discovery.

In this multidisciplinary study, we used molecular dynamics simulations to analyse the behaviour of the 216-loop of hPIV-1 HN and compare it to that of hPIV-3 HN. While the hPIV-3 HN 216-loop can fluctuate between open and closed conformations, hPIV-1 HN loop is predominantly closed in the simulations. We then designed and synthesised a series of Neu5Ac2en derivatives that incorporate C4-functionalized triazoles. Using neuraminidase inhibition, hemagglutination and cell-based infection inhibition assays, we showed that the size of the C-4 substituent has an effect on the inhibition of hPIV-1 HN activities. We have also explored a range of inhibitors with small substituents at C-4 to specifically target hPIV-1 HN. Finally, we looked at the potency of C-4 epimers of our lead compounds as well as the effect of the position of the triazole's substituent on the ring.

This study demonstrates the relation between substituent size and inhibitor selectivity to hPIV-1 HN which seems to be dictated by the closed conformation of the 216-loop. We also report that one of our designer compounds reaches levels of inhibition comparable to the most potent hPIV-1 inhibitor published to date.

(81) How sweet are our gut beneficial microbes?

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Glycosylation is the most common post translational modification of proteins in nature and is essential to various biological and physical processes. Protein glycosylation in prokaryotes, and especially pathogens, has attracted much attention in recent years due to the role of glycans in adhesion, colonisation or virulence. In contrast, and despite the increasing interest in gut microbiota, not much is known on protein glycosylation in commensal bacteria.

Adhesion of gut commensals to the host tissue is the first step to succesful colonisation and is mediated by bacterial cell-surface adhesins. Recent studies established *Lactobacillus reuteri* as a model organism to study the evolution and host specialisation of gut symbionts. *L. reuteri* colonises the gut of various vertebrates and expresses adhesins mediating the interaction of the strains to their specific host. In particular, pig and human isolates that colonise the small intestine produce mucus binding proteins (MUB), whereas rodent isolates that colonise the forestomach epithelium express a serine-rich repeat (SRR) protein, previously identified in pathogens. Using a combination of lectin affinity studies, gas chromatography, mass spectrometry (MALDI-ToF, ESI-MS) and atomic force microscopy (AFM), we have obtained evidence that the main *L. reuteri* adhesins are glycosylated. Our preliminary data suggest that *L. reuteri* glycosylates proteins using short, neutral glycan chains with MUB carrying glycans with terminal galactose and SRR carrying di-GlcNAc moieties. We are also investigating by mutagenesis the molecular pathways leading to the glycosylation of these adhesins, including the the accessory secretion system ($SecA_2/Y_2$) gene cluster responsible for SRR glycosylation and glycosyltransferases putatively involved in glycosylation of MUB.

Taken together our data suggest that the *L. reuteri* $SecA_2/Y_2$ cluster found in pig and rodent isolates is dedicated to the glycosylation of SRRs with glycans shorter than those found in pathogens, and that there is at least one additional glycosylation system responsible for the glycosylation of other adhesins.

(82) Plasmodium falciparum rosetting domain recognizes ABH histo-blood group antigens in a type specific manner

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The ability to use infected erythrocyte to adhere to endothelial cells (cytoadherence) and to capture uninfected erythrocyte (rosetting) are main virulence factors of *Plasmodium falciparum*. Globally, the infection established by this pathogen is the leading cause of death by severe malaria. From a molecular point of view, it is well known that cytoadherence and rosetting processes are mediate by adhesins. Evidences link the binding of adhesin Duffy Binding Like1-α (DBL1α) domain of the Erythrocyte Membrane Protein 1 (PfEMP1) to the ABH histo-blood antigens with formation of rosettes. Inspired by this very close relationship between the disease susceptibility and individual blood type, here we investigate the structural requirements involved in the interaction of DBL1α with A, B and H histo-blood determinants, focusing on its subtypes.

We have confirmed the preference of DBL1α for A epitopes using the recombinant domain from FCR3S1.2 strain, which was shown to be independent from the NTS region as reported for DBL1α domain from Palo Alto strain. Notably, DBL1α interacts with ABH epitopes in subtype specific manner presenting a remarkable affinity for type 2 structures, characterized by the core Fucα1-2Galβ1-4GlcNAcβ1, particularly the A type 2 epitope. Type 1 and fucosylated variants of ABH epitopes revealed inherent relative low affinities, but due to different structural features: while the specific glycosidic linkage at C4 of β-GlcNAc residue, in type 1, seems to limit interaction, the additional α-Fuc promotes steric effects which hamper the binding.

The contacts made by DBL1α binding pocket and the ABH histo-blood groups were mapped by theoretical methods and supported by NMR experiments, and highlights structural differences between DBL1α from the virulent FCR3S1.2 and Palo Alto strains. In fact, there is an increment of 14 residues in DBL1α sequence from FCR3S1.2 strain compared to Palo Alto, which adds polar residues that may effectively interact with blood group epitopes, mainly by their core portion. In summary, data presented here contribute notably by clarifying the molecular mechanism that regulates host infection by *P. falciparum*, but also by guiding the search for protein inhibitors and the development of new malaria prophylaxis.

(83) The Price of Flexibility – A Comformational Study on Oxepanes as Mannose Mimetics

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The thermodynamic outcome of the interaction of a flexible ligand and a rigid binding pocket of a receptor can rarely be dissected quantitatively into enthalpic and entropic contributions. In the present study, a polyhydroxylated oxepane was studied as a ligand for the mannose-specific lectin FimH. FimH is located at the tip of type 1 pili of uropathogenic *E. coli* and plays an essential role in the first step of the bacterial infection. Competitive binding assays and isothermal titration calorimetry (ITC) indicated an approx. ten-fold lower affinity for 3-*n*-heptyloxy oxepane compared to *n*-heptyl α-D-mannopyranoside, exclusively resulting from a loss of conformational entropy. Further investigations by solution NMR, X-ray crystallography and molecular modeling revealed that the oxepane ligand establishes an identical H-bond network compared to the mannoside prototype, but for the price of an high entropy penalty due to loss of its high conformational flexibility. These results underscore the importance of having access to the complete thermodynamic profile of a molecular interaction to "rescue" ligands from entropic penalties with otherwise perfect fit to the protein binding site.

(84) Structural studies of the lipopolysaccharide produced by plant pathogen Xylella fastidiosa

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Xylella fastidiosa is a gram-negative, xylem-inhabiting, vector transmitted, bacterial pathogen that causes serious diseases in economically important crops, such as Pierce's Disease of grapevines, citrus variegated chlorosis, and almond leaf scorch.[1]

The bacterium produces copious amounts of biofilms consisting of exopolysaccharides (EPS) and lipopolysaccharides (LPS) which occludes xylem vessels and impedes water flow within the vine. In the case of *X. fastidiosa*, the O-chain polysaccharide of the LPS is regarded as a major factor of pathogenicity. Interestingly, a mutation made in the *wzy* gene, which encodes an O-antigen polymerase, caused a marked defect in biofilm formation.[2]

The aim of this study was to purify and structurally characterize LPS, specifically focusing on the O-antigen moiety. Detailed structural analysis, based on monosaccharide analysis, methylation analysis, mass spectrometric and 1D/2D NMR spectroscopic studies on O-antigen isolated from *X. fastidiosa* wild type, revealed that the major polysaccharide

was composed of a linear (α1→2)-L-rhamnan and the minor polysaccharide consisted of α-L-rhamnan backbone substituted with either two or one β-D-Xyl residues.

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(85) Global Mapping of O-Glycosylation of Human Herpesviruses

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Herpesviruses are among the most complex and widespread viruses, infection and propagation of which depend on envelope proteins. These proteins serve as mediators of cell entry as well as modulators of the immune response and are attractive vaccine targets. Although envelope proteins are known to carry glycans, little is known about the distribution, nature, and functions of these modifications. This is particularly true for O-glycans; thus we have recently developed a bottom up mass spectrometry-based technique for mapping O-glycosylation sites on herpes simplex virus type 1. We found wide distribution of O-glycans on herpes simplex virus type 1 glycoproteins and demonstrated that elongated O-glycans were essential for the propagation of the virus. Here, we applied our proteome-wide discovery platform for mapping O-glycosites on representative and clinically significant members of the herpesvirus family: varicella zoster virus, human cytomegalovirus, and Epstein-Barr virus. We identified a large number of O-glycosites distributed on most envelope proteins in all viruses and further demonstrated conserved patterns of O-glycans on distinct homologous proteins. Because glycosylation is highly dependent on the host cell, we tested varicella zoster virus-infected cell lysates and clinically isolated virus and found evidence of consistent O-glycosites. These results present a comprehensive view of herpesvirus O-glycosylation and point to the widespread occurrence of O-glycans in regions of envelope proteins important for virus entry, formation, and recognition by the host immune system. This knowledge enables dissection of specific functional roles of individual glycosites and, moreover, provides a framework for design of glycoprotein vaccines with representative glycosylation.

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(86) C-mannosylation of Thrombospondin Type 1 Repeats in Apicomplexan Parasites

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The phylum Apicomplexa contains many parasites of medical and veterinary importance including the notorious malaria agent *Plasmodium falciparum* as well as important foodborne pathogens such as *Toxoplasma gondii*. Apicomplexa are obligate intracellular parasites and require a distinctive set of adhesins for host cell attachment and active invasion. Amongst these, proteins containing one or several thrombospondin type 1 repeats (TSRs), such as proteins of the conserved thrombospondin-related anonymous protein (TRAP) family, play a central role.

In animals, TSRs carry two distinct types of glycosylation: C-mannosylation and O-fucosylation. O-fucose is transferred by the protein O-fucosyltransferase 2 (POFUT2) to a conserved serine or threonine residue in the consensus sequence $CX_{2-3}(S/T)CX_2G$, and may be further extended by the β1,3-glucosyltransferase B3GLCT to form the epitope Glcβ1,3Fucα-O-Ser/Thr. In addition, α-mannose can be attached via a carbon-carbon linkage to the indole ring of the first tryptophan in the consensus sequence WXXW/C, a reaction mediated by the C-mannosyltransferase DPY-19 in *Caenorhabditiselegans* (Buettner *et al.*, 2013). Homologues of the gene encoding POFUT2, B3GLCT and DPY-19 can be found in the genome of various apicomplexan parasites.

CRISPR-Cas9-generated CHO cell lines deficient in C-mannosylation were used for expression of TSR-containing adhesins of *P.falciparum* and *T. gondii* with or without the parasite DPY-19 homologues. The proteins were then purified, digested with proteases, and the resulting glycopeptides were analyzed by high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) and tandem mass spectrometry. These analyses demonstrated that the DPY-19 homologues of *P. falciparum* and *T. gondii* were able to C-mannosylate *P. falciparum* TRAP and TRAP-related *T. gondii* micronemal protein 2 (MIC2), respectively. Interestingly, *T. gondii* DPY-19 can C-mannosylate the first tryptophan of a WXXW and of a WXXC motif. Hence, *T. gondii* DPY-19 seems to exhibit broader substrate specificity than *C. elegans* DPY-19 that has specificity for the first tryptophan in WXXW motifs only. Finally, as already observed for several metazoan proteins, C-mannosylation had a beneficial effect on MIC2 secretion, suggesting that the modification stabilizes TSR-containing proteins.

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(87) Histo-blood group antigen presentation is critical for norovirus VLP binding to glycosphingolipids in membranes

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Virus-glycan interactions at the cell membrane are often key factors for virus uptake and internalization into host cells. The orientational flexibility of membrane associated glycans is restricted as compared to their soluble forms. How this restriction affects virus binding to the lipid membranes is poorly understood. Here, we employed equilibrium fluctuation analysis using total internal reflection fluorescence microscopy (TIRFM) based assay and molecular dynamics (MD) membrane simulations in an effort to investigate the consequences of such presentation effects. The system studied was histo-blood group antigen (HBGA) epitopes of membrane bound glycosphingolipids (GSLs) interacting with human GII.4 norovirus-like particles (NVLPs). Our results give an explanation model as to why there is a clear difference in experimental results between the binding properties of Ast6139 GII.4 NVLPs to membrane bound HBGA glycans and to the same glycans in solution as studied with saturation transfer difference (STD) NMR. In particular, HBGA Lewis x demonstrated a clear saturation difference in complex with NVLPs in solution but did not show any attachment in the membrane associated form. Our MD data indicate that this lack of binding is due to the obscured orientation of the Lewis alpha1,3-fucose when presented as membrane associated glycan. Our results contribute to a general understanding of protein-carbohydrate interactions on the membrane surface and provide valuable insights into the importance of glycolipid presentation effects in the context of virus binding.

(88) The effect of tandem-repeat galectins on morphology of Escherichia coli and their adhesion to host cells

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The gastrointestinal mucosa is exposed to a large number of commensal microbes. It is known that several different galectins are expressed by intestinal epithelial cells. We detected an abundant amount of galectin-4 and galectin-9 in the secretion from freshly isolated intestine. Therefore, commensal bacteria colonized on the intestinal mucosal surface could be in contact with galectins existing in the luminal space. Recent studies have identified the ability of galectin-3, -4 and -8 to bind to and kill bacteria expressing blood group antigen (self antigen). The consequence of bacteria containing non-self antigen encountering galectins is not known. In this study, we used *Escherichia (E.) coli* O19ab as a model and found that galectin-4, -8 and -9 could bind to *E. coli* O19ab through protein-carbohydrate interactions. In addition, endogenous galectin-9 could be detected on the surface of *E. coli* O19ab present in the intestinal lumen. Tandem-repeat galectins did not affect *E. coli* O19ab survival, but induced bacteria to form filaments, in which cells were connected end-to-end in a chain, and galectins were highly enriched at the cell-cell junctions. It seems that this may be due to cell membrane attachment and membrane fusion. In addition, *E. coli* O19ab treated with tandem-repeat galectins increased their adhesion to intestinal epithelial cells. Our study demonstrates that extracellular galectins can affect morphology of *E. coli* and their adhesion to the intestine.

(89) Trypanosoma cruzi chronic infection in Galectin-8 knock out mice

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Galectins are lectins with affinity for beta-galactosides and are involved in multiple biological activities. Galectin-8 (Gal-8) is widely distributed through different tissues both in normal and pathological conditions. It participates in cellular adhesion, apoptosis, T cells proliferation, pro-inflammatory events, clearance of intracellular bacteria, etc. *Trypanosoma cruzi*, is a protozoan intracellular parasite that induces chronic cardiomyopathy, the main pathological finding in Chagas disease. This inflammatory malignancy (diffuse mononuclear cells, fibrosis and scarce parasites) affects 30% of infected patients and manifests 20 years after *T. cruzi* infection. The knowledge on Gal-8's role in the infection is scarce. To analyze the possible role of this galectin in *T. cruzi* infection, male Gal-8 KO and C57BL/6J (WT) mice were infected with 10^4 Ac *T. cruzi* strain (DTU I) trypomastigotes and sacrificed 4 months pi. Tissue sections were obtained from heart, liver and skeletal muscle, stained with H&E and Masson's trichrome and evaluated for extent of inflammation, fibrosis and parasite density. Higher inflammation in heart (p=0.0119), skeletal muscle (p=0.0232) and liver (p=0.0155) was observed in Gal-8KO mice compared to infected WT and normal, ANOVA test statistical analysis was employed. The extent of fibrosis in myocardium was similar between infected groups. In skeletal muscle the number of parasitized cells was higher in Gal-8KO mice than in WT mice (P 0.0259), which shows higher

susceptibility to *T. cruzi* infection. The correlation between the absence of Gal-8 and the significant increase of inflammation in the different tissues analyzed, suggests a role for Gal-8 in chronic inflammation modulation.

(90) Fucosylation contributes to Cholera toxin intoxication, even in the presence of GM1

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Introduction: Cholera toxin subunit B (CTB) interacts with cell surface receptors for internalization of cholera holotoxin (CT). Cholera is a water-borne disease and CT intoxicates the intestinal cells. We have recently identified that in colon epithelial cell lines CTB utilizes fucosylated glycoproteins as its interacting receptors. We also reported that colonic cells express low levels of the canonical receptor, glycolipid GM1, when compared to other cell types. In the light of our recent findings, the understanding the pathway of CTB internalization pathways in colon epithelial cells in the presence of GM1 becomes important. Therefore, we wanted to determine the effect of cellular GM1 levels on CTB surface binding, internalization, and endocytosis in colon epithelial cell lines. Methods: All of our experiments were performed in colon epithelial cell lines (Colo205, LS174T and T84) with variable concentrations of GM1 and/or other glycosylation inhibitors. CTB cell surface binding was determined using flow cytometry in Colo205 cells and a cell surface ELISA in LS174T and T84 cells. An In-Cell ELISA (ICE) protocol was developed to measure internalization of CTB. CT activity was determined by cellular cAMP levels in LS174T and T84 cells. Results: We observed a GM1 dose-dependent improvement of CTB uptake in all three cell lines, with addition of 20 μg/ml GM1 to cell culture medium improving CTB binding by 5-8 fold. Addition of GM1 (20 μg/ml) increased internalization of CTB by 2-2.5 fold and cAMP levels by ~5 fold. Upon Nontheless, upon addition of 20 μg/ml GM1, free L-fucose, but not D-fucose or other free sugars, was able to inhibit CTB uptake and internalization. Chemical inhibitionor of cell surface fucosylation (2F-Fucose) also blocked reduced GM1-mediated CTB cell surface binding, internalization and induced cAMP levels, even in cells supplemented with GM1. Conclusions: GM1 improves CTB uptake, internalization and CT-activity in the three cell lines. Chemical inhibition of fucosylation using 2F-Fucose or by addition of free L-fucose partially blocked CTB activity in presence of GM1. Taken together, these data indicate that CTB internalization in colon epithelial cells is dependent on fucosylated glycoproteins along with GM1. While GM1 addition improves CTB cell surface binding, CTB still utilizes fucosylated structures for binding and internalization in colon epithelial cells.

(91) Examination of the protease inhibitor ecotin and N-linked glycosylation, an insight into protein protection in the protease rich environment of the oral cavity

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Protein glycosylation in bacteria was first described in the foodborne pathogen Campylobacter jejuni. Interestingly, all Campylobacter species possess an N-linked protein glycosylation (pgl) pathway, but only species that inhabit the oral cavity and have been implicated in the onset of periodontitis (i.e Campylobacter rectus and Campylobacter showae) encode an orthologue of the serine protease inhibitor, ecotin, within their pgl locus. Serine proteases such as neutrophil elastase are highly abundant in the oral cavity and play a significant role in killing pathogenic bacteria. In C.jejuni, we previously demonstrated that modification with N-linked glycans protects glycoproteins from proteolytic attack by chicken cecal proteases and enhances bacterial survival in this environment; however, N-glycosylation alone may not be sufficient to provide full protection from proteases in the oral cavity.

We investigated if ecotin orthologues from oral campylobacters can protect against serine proteases. Ecotins from C. rectus and C. showae were overexpressed and purified from E. coli as C-terminal hexa-histidine-tag fusions. Initial protease protection assays using CmeA from C. jejuni as a substrate demonstrated that both campylobacter ecotins protect CmeA from trypsin degradation whereas CmeA was degraded in the absence of ecotin. We tested ecotin in a fluorescence resonance energy transfer (FRET) assay based on a peptide that contains specific protease cleavage sites flanked by two fluorescentmolecules. We could demonstrate that cleavage of the FRET peptide by factor Xa and elastase led to an increase in fluorescence that was inhibited by the campylobacter ecotins as well by the E.coli ecotin (positive control). Moreover, protection by campylobacter ecotins was equivalent to the E. coli ecotin indicating that the tested ecotins have similar protective properties. Since, ecotins are located in the periplasmic space of Gram-negative organisms, we are currently determining if ecotins could inhibit the campylobacter major periplasmic serine protease/protein chaperone, HtrA. It will be interesting to examine the interaction between ecotins and self-proteases and their respective roles in the folding and recycling of N-glycosylated proteins in the periplasmic space of oral Campylobacter species.

(92) NMR Structure of Streptococcal IgA-Fc Receptor Siglec-5 Binding Domain

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Group B streptococcus (GBS), also known as *Streptococcusagalacticae*, is a bacterium that colonizes the gastrointestinal and genitourinary tracts of healthy individuals. Despite universal screening and intrapartum prophylaxis of pregnant women, the organism remains an important cause of serious infections among newborns and an emerging pathogen in the elderly and immunocompromised populations.A criticalGBS virulence determinant is its sialic acid-capped polysaccharide capsule,which impairs opsonophagocytosis and can engage humansialic acid-binding Ig-like lectins (Siglecs) on leukocytes to dampen immune responses.

It was recently shown that some GBS serotypes are capable of binding to human Siglec-5 in a sialic acid-independent manner [1]. This interaction was found to be mediated by a 125 kDa cell-surface protein called IgA-Fc receptor (also named β protein, or β antigen) binding to the V-set domain of Siglec-5. The segment responsible for Siglec-5 binding was mapped to the N-terminal region distinct from the IgA-Fc binding domain [2]. The Siglec-5 binding domain had no known homologs in terms of amino acid sequence, and the molecular mechanism of the interaction remained unknown.

We have determined the solution NMR structure of the N-terminal fragment of the IgA-Fc receptor, containing the Siglec-5 binding domain. The structure consists primarily of a three-helix bundle, with geometry very close to that of the IgG-Fc binding domains of staphylococcal protein A, despite a very low sequence similarity. In conjunction with the previously determined crystal structure of the V-set and C2-set domains[3] of human Siglec-5 it is expected to provide an insight into the interaction mechanism.

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(93) Innate Immune Galectin Targets Sialylated Microbe

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Many microbes have developed the ability to evade host adaptive immunity by coating their surface with mammalian-like antigens, a phenomenon known as molecular mimicry. Recent studies suggest that a class of innate immune factors called galectins fill this gap in adaptive immunity by specifically targeting microbes that utilize molecular mimicry. However, while galectins typically recognize mammalian-like antigens that terminate in galactose, many microbes cap galactose residues with sialic acid, a modification previously thought to inhibit galectin-glycan interactions. As a result, we sought to determine whether innate immune factors exist that possess the ability to target microbes that utilize sialylated forms of molecular mimicry. Using a combination of mammalian and microbial glycan microarrays, we found that galectin-8 (Gal-8) uniquely recognizes sialylated glycans with high affinity, suggesting that Gal-8 may be distinctively poised to provide innate immunity against sialic acid-based molecular mimicry. Consistent with this hypothesis, Gal-8 not only recognized sialylated glycans in a microarray format, but also bound the sialylated Group B Streptococcus (GBS) strain A909, which resulted in rapid microbial death. While these results suggest that Gal-8 recognizes and kills GBS A909 through a sialic-acid-dependent process, Gal-8 displayed a very similar ability to kill a mutant strain of GBS (A909 delta) that fails to generate a sialylated capsule. Further analysis of the individual domains of Gal-8 demonstrated unique and complementary binding and killing activity of each domain. While the N-terminal domain (Gal-8N) intrinsically bound and killed GBS A909, the C-terminal domain (Gal-8C) failed bind or impact GBS A909 viability. In contrast, Gal-8C readily bound and killed the non-sialylated GBS A909 delta, yet failed to recognize or kill GBS A909. Given the ability of many microbes to readily alter their surface sialylation, these results suggest that Gal-8 uniquely evolved to combat microbes that seek to avoid host immunity by self-modulating surface sialylation levels. These results also provide the first example of an innate immune factor that possesses the ability to specifically and intrinsically recognize and kill a sialylated microbe, providing a unique example of innate immunity against sialic acid-based molecular mimicry.

(94) Clostridium difficile chemotaxes towards intestinal mucus and forms biofilms in a complex community

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Background: *Clostridium difficile* infection (CDI) is the most common cause of bacterial-induced diarrhea and severe colitis in the United States. Studies have shown that antibiotic

disruption of the gut microbiota creates a favorable niche for *C. difficile* spore germination and proliferation. In order to reach the epithelium, gastrointestinal (GI) pathogens such as *C. difficile* must associate with the intestinal mucins. As mucus is among the first lines of epithelial defense, this information may hold the key for *C. difficile* host colonization and be a potential preventative treatment target. Methods & Results: Our data demonstrate that *C. difficile* R20291, ATTC 630 and three clinical isolates are capable of chemotaxis towards intestinal mucus derived from both stool and the mucus-secreting cell lines HT29-MTX and LS174T. *C. difficile* chemotaxes specifically toward cleaved O-linked glycans and mucin monosaccharides. *C. difficile* did not migrate towards sialic acid or the amino acid cysteine. Using CFDA-SE tagged bacteria we demonstrated that *C. difficile* is capable of adhering to stool mucus in a glycan dependent manner. Computational modeling of *C. difficile* adhesion proteins demonstrated the ability of several proteins to bind to mucin glycan structures, providing further evidence for *C. difficile* mucin adherence. Adherence of *C. difficile* to mucin glycans promoted the formation of biofilms. To assess chemotaxis and biofilm formation in the setting of a complex community, mucin coated coverslips were suspended in bioreactors containing antibiotic-treated human stool supplemented with *C. difficile*. Fluorescent in situ hybridization (FISH) revealed that *C. difficile* was present with other microbes in a mucus-associated biofilms and16S sequencing was used to determine the composition of the biofilm. Importantly, *C. difficile* was present in biofilms in surgical resections from patients with CDI, confirming the role of intestinal mucus in *C. difficile* colonization *in vivo*. **Conclusions**: Collectively, these studies suggest that *C. difficile* adheres to mucin glycans and adherence promotes biofilm formation. Adherence to mucins and formation of biofilms may allow *C. difficile* to efficiently deliver toxins in close proximity to the host, thus providing a potential mechanism for persistent colonization and infection in CDI.

(95) A Novel Periplasmic Mannan-Binding Protein Involved in the Synthesis of Lipomannan in Mycobacteria

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The mycobacterial cell envelope has a complex multilaminate structure containing an inner membrane, a peptidoglycan-arabinogalactan-mycolic acid layer, and an outer membrane. Phosphatidylinositol (PI)-anchored mannans such as PI mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) are glycolipids critical for the structural integrity of the cell envelope. Sequential additions of mannoses to PI produce PIMs, generating two major forms: AcPIM2 and AcPIM6, carrying two and six mannose residues, respectively. We previously deleted the *pimE* gene in the non-pathogenic model *Mycobacterium smegmatis*. Δ*pimE* was unaffected in LM/LAM biosynthesis. However, this mutantfailed to produce AcPIM6, and instead, accumulated AcPIM4 intermediates, suggesting that PimE is the fifth mannosyltransferase of the PIM biosynthetic pathway. In the current study, we found that Δ*pimE* formed significantly smaller colonies than wild type (WT) on standard Middlebrook 7H10 agar. Taking advantage of this growth defect, we isolated spontaneous suppressor mutants of Δ*pimE* that reverted to WT colony size. One suppressor mutant accumulated significantly smaller LM, and whole genome sequencing revealed a point mutation in a previously uncharacterized gene that we designated as *spe2*. The *M. smegmatis spe2* complemented this suppressor mutant, restoring the original phenotypes of Δ*pimE* such as small colony size and normal LM size. Next, we created a *spe2* deletion (Δ*spe2*) mutant in the WT background and showed that the Δ*spe2* mutant produced smaller LM as seen in the suppressor mutant. Bioinformatic analyses suggest Spe2 is an extra-cytoplasmic protein conserved throughout the Corynebacteriaceae family. Protease accessibility and detergent extractability of Spe2 in intact cells were consistent with the interpretation that Spe2 is not surface-exposed. Finally, purified Spe2 binds to an LM intermediate in an ELISA assay for lipid-protein interaction. Together, these data suggest that Spe2 is a periplasmic mannan-binding protein critical for controlling the mannose chain length of LM/LAM.

(96) A Y161F hemagglutinin substitution improves yields of a 2009 H1N1 influenza A vaccine virus in cells by increasing their binding affinities to alpha 2,3-linked and 2,6-linked sialic acid receptors

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Influenza is responsible for a substantial disease burden worldwide and vaccination remains the primary strategy for its prevention and control. Most human influenza vaccines are prepared from viruses grown in embryonated chicken eggs which can be selected for antigenically distinct variants. Combined with possible egg-based allergic reactions, this has hastened the development of cell-based vaccines. However, wild type vaccine seed viruses usually do not grow efficiently in mammalian cell lines. Recently, by screening a large mutant library of hemagglutinin for 2009 H1N1 virus, one mutant (Y161F) had a 1,000 fold higher infectivity titer in MDCK and Vero cells when compared to the wild type A (H1N1)pdm09xPR8 virus. The total protein yield of the Y161F mutant was 4.6 times that of the wild type in MDCK cells. Viruses were cultured in MDCK for 3 passages and confirmed to maintain a similar antigenicity (<2-fold

difference) as the wild type virus. To understand the impacts of Y161F in the viral replications, we performed viruses-glycan receptor binding assays using Octet® K2 System. Results showed that, compared to A(H1N1)pdm09, the Y161F mutant had significantly higher avidities to all of four testing synthetic N-linked glycans than the wild type virus did, including two with alpha 2,3-linked sialic acids (SA), and two with alpha 2,6-linked SA. This study suggested that a Y161F hemagglutinin substitution improves its binding affinity to alpha 2,3-linked and 2,6-linked sialic acid receptors, which could potentially be responsible for the increase of replication efficiencies for 2009 H1N1 virus in cells.

(97) Mutations in hemagglutinin of H6N6 influenza A virus changed glycan receptor binding properties when being transmitted from avian to swine

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Influenza A virus (IAV) causes both seasonal and pandemic outbreaks and continue to pose great threats to public health. In addition to human, IAV has a wild range of hosts including avian, swine, canine, equine, and sea mammals. Novel IAVs emerging at the human-animal interface, especially the human-swine interface, pose pandemic threats due to lack of the pre-existing immunity in human population. In 2010, an H6N6 IAV emerged in southern China, and since then, it has caused sporadic infections among swine. Phylogenetic analyses and molecular characterization suggested this H6N6 swine IAV was avian origin, and, in the hemagglutinin (HA) protein, has 222V and 228S whereas avian IAVs have amino acids 222A and 228G. To illustrate the potential impacts of these mutations in host adaptation for H6N6 IAV when being transmitted from avian to swine, we generated three mutants by reverse genetics, and compared the binding affinity of these mutants with wild type H6N6 swine IAV to *N*-acetylneuraminic acid–α2, 6–linked galactose (NeuAcα-2,6Gal; human-like receptors) and *N*-acetylneuraminic acid–α2, 3–linked galactose (NeuAcα-2,6Gal; avian-like receptors). The viruses-glycan receptor binding assays were performed by an Octet® K2 System with six synthetic glycans, including three NeuAcα-2,6Gal glycans and three NeuAcα-2,6Gal glycans. Results showed that rgH6N6×PR8-222V/228S (swine IAV-like) binds to both NeuAcα-2,6Gal and NeuAcα-2,3Gal; the binding affinities of this virus to NeuAcα-2,3Gal is much stronger than those to NeuAcα-2,6Gal. However, the mutants with 222V/228G (avian IAV-like) or 222A/228G (avian IAV-like) bound solely to NeuAcα-2,3Gal. In summary, this study suggested that mutations A222V and G228S changed the receptor binding affinities of H6N6 IAV and could help this virus adapt to swine when being transmitted from avian to swine.

(98) HUMAN ADENOVIRUS TYPE 5 MODIFIES FUCOSYLATION IN A CELL MODEL OF HUMAN LUNG EPITHELIUM

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The carbohydrate moieties of glycoconjugates modulate important cellular processes (eg. differentiation, proliferation or pathogen cell-host interactions), and their synthesis and structure is subject to genetic and environmental regulatory events that modify the activity of enzymes and of other proteins involved in glycosylation. The absence or presence of core or terminal fucose is an example of these modifications. Glycoprotein fucosylation is implicated in a wide variety of fundamental physiological processes as diverse as memory and learning, or cancer and immunity.

Few studies have addressed the modulation of glycosylation by viral infections and its importance for the infectious process. Infection with CMV, VZV and other herpes viruses increases the expression of fucosylated epitopes in infected cells that are considered to favor the dissemination of viral progeny.

In this work we have established, using lectin profile (AAL, UEA-I, LCA, SNA and MALII) and carbohydrate metabolic labeling for fucose and sialic acid, that infection with human adenovirus 5 (Ad5), a common infectious agent of airway epithelia and a vector used in gene and anti-cancer therapy, increases surface fucosylation of A549-infected cells measured by Fucose alkyne. Particularly, adenovirus infection induced 20% increase in Fuc α1-2 moieties assessed with UEA-I lectin.

Also, we have established that increased fucosylation is dependent on the expression of viral genes. Characterization of the specific glycoproteins responsible for the increase in surface fucosylation is discussed.

(99) The oligosaccharyltransferase subunit DC2 mediates the association between the STT3A and Sec61 complexes

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Metazoan organisms assemble two oligosaccharyltransferase (OST) complexes that are composed of a catalytic subunits (STT3A or STT3B), and a shared set of accessory subunits as well as isoform specific subunits. The STT3A isoform lies close to Sec61 complex and mediates the co-translational glycosylation of the nascent polypeptide as it enters the endoplasmic reticulum. The mechanism responsible for localization of the STT3A complex adjacent to the Sec61complex has not been defined. Here, we show that DC2 and KCP2 are complex-specific subunits of the STT3A complex. A CRISPR/Cas9 generated HEK293 derived DC2 knockout cell line has a severe defect in glycosylation of

STT3A dependent substrates that phenocopies disruption of the STT3A gene. STT3A complexes are more readily solubilized in digitonin-high salt solutions in DC2 (-/-) cells than in control cells. Ribosome associated membrane protein (RAMP) complexes are composed of a translating ribosome, the Sec61 complex, the STT3A and TRAP complexes. Elimination of the DC2 protein resulted in the loss of the association between the STT3A and Sec61 complexes as monitored by blue-native PAGE. DC2 deletion mutants lacking 5 residue segments were expressed in DC2 (-/-) cells and assayed for incorporation into the STT3A complex by BN-PAGE analysis and for the ability to restore glycosylation of prosaposin by pulse labeling. BN-PAGE analysis revealed that the C-terminal lumenal tail of DC2 is essential for incorporation into the STT3A complex. DC2 mutants lacking the C-terminal tail, or lacking residues in a lumenal loop were unable to promote cotranslational glycosylation of prosaposin. We conclude that the DC2 subunit of the STT3A complex is responsible for mediating the association between the STT3A complex and the protein translocation channel.

(100) An alternative N-linked protein glycosylation biosynthesis pathway in Campylobacter fetus utilizing a unique lipid intermediate

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The N-linked protein glycosylation (Pgl) pathway is conserved throughout the Campylobacter genus and is involved in a multitude of cellular functions. In Campylobacter fetus, the pathway produces two unique hexasaccharides: GlcNAc-(Glc)-GlcNAc-GalNAc-GalNAc-diNAcBac (where diNAcBAc is 2,4-diacetamido-2,4,6-trideoxy-D-Glc) and GlcNAc-(GlcNAc)-GlcNAc-GalNac-GalNAc-diNAcBac, which occur in a ratio of 1:4 respectively. These hexasaccharides are assembled onto undecaprenylphosphate (UndP) on the cytoplasmic side of the inner membrane, flipped into the periplasmic space and then transferred onto the asparagine residue of the bacterial glycosylation sequon D/E-X1-N-X2-S/T (where X1/X2 are not proline) by the oligosaccharyltransferase, PglB. In silico analysis of the genes adjacent to the pgl gene cluster identified a putative bacteriophage glucosylation operon, gtrABC, typically involved in lipopolysaccharide O-antigen modification. The operon encodes three enzymes: GtrA, a flippase for the UndP-Glc precursor; GtrB, an UndP-glucosyltransferase; and GtrC, an O-antigen-specific glucosyltransferase. We hypothesized that the gtr operon in C. fetus is responsible for the addition of Glc to the pentasaccharide backbone coupling the unique Und-P-sugar intermediate. We demonstrated that the Gtr enzymes preferentially transfer Glc from UDP-Glc to UndP using an in vitro UDP-Glo™ assay (Promega) that allows the quantification of UDP release by GtrB. In addition, co-expression of the C. fetus gtr operon, the components of the pgl operon and an N-glycan acceptor protein in E. coli resulted in the formation of a glycopeptide with a parent mass that is consistent with the C. fetus hexasaccharide with the Glc branch. An in vitro reaction using GtrB resulted in a major product with a mass corresponding to undecaprenyl-phosphate-hexose. The assembly of an N-glycan through the combination of en bloc transfer from a lipid-diphosphate-oligosaccharide together with addition of a lipid-phosphate-monosaccharide branch is homologous to N-glycosylation systems found in all three domains of life suggesting further conservation of N-glycosylation mechanisms. Also, our findings indicate that the components of the bacteriophage gtrABC operon have been subverted by C. fetus for the transfer of a glucose residue from Und-P-Glc to the pentasaccharide synthesized by enzymes encoded within the pgl operon and therefore suggest a unique mechanism for N-glycan biosynthesis in bacteria.

(101) N-Glycan transition of early developmental Oryza sativa seedlings exposed by silver nanocolloids

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"Nanomaterial" is a general term for small substance, and that is 1-100 nm in diameter. In particular, silver nanomaterials have been developed and consumed mainly as the antibacterial products in the world because of those antibacterial activities. However, there is hardly information about potential risks of silver nanomaterials to animals and plants. On the other hand, "*N*-glycans" play important roles in various biological phenomena, and the structure and expression are sensitive to ambient environmental changes. Therefore, As a first model for assessment of the influence of silver nanomaterials, we focus on the correlation of *N*-glycan structures and silver nanomaterials in plant, and analyzed the *N*-glycan structures of *O. sativa* seedling exposed by silver nanocolloids (SNCs).

O. sativa seeds were immersed in SNCs suspension for 96h. The results of SNCs exposure, it was confirmed that the root was seriously damaged, while the shoot was not affected. The results of size-fractionation HPLC analysis, three peaks were increased in the SNCs exposed root, and these three peaks were further separated by the reversed-phase HPLC analysis. The results of reversed-phase HPLC analysis, five major peaks were obtained. As a noteworthy point, one peak was eluted at the flow through position. The results of MALDI-TOF mass spectrometry and sequential

enzyme digestion, the flow through peak was assigned as free-*N*-glycan structure with one GlcNAc residue to the reducing end. All remaining other *N*-glycan structures in *O. sativa* root were determined by retention time of size-fractionation and reversed-phase HPLC, and it was confirmed that the ratio of plant complex type *N*-glycan in the SNCs exposed root was up to six fold higher than that of control. Interestingly, the ratio of free-*N*-glycan was also dramatically increased in the SNCs exposed root in comparison with control. Our results suggest that the transition of plant complex type *N*-glycan including free-*N*-glycan was caused by the abnormalities of *O. sativa* development.

(102) Primary structure determination of a blood group B-specific lectin purified from Streptomyces sp. 27S5 reveals insight into its mechanism of expression and unique structural features

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A hemagglutinin produced by *Streptomyces sp*. 27S5 (SHA) was characterized in 1970's. At that time SHA was purified from 15 L of culture supernatant of *S.* sp. 27S5 by gum arabic affinity chromatography with a yield of 60 mg and 64% recovery. About 200 mg of SHA was purified for structural-functional studies, which revealed that the small ~11 kDa SHA protein had rare blood group-B hemagglutination specificity, a tryptophan-rich nature, and two carbohydrate-binding sites. When SHA, kept frozen since 1975, was re-analyzed in 2014, it was found to be intact with activity and a molecular mass of 13,314.67 Da. We used mass spectrometric proteomics methods and genomic databases to solve the primary structure of SHA. Although the original strain isolated in Japan was lost, *Streptomyces* genomic databaseshad recently grown so vast that it became possible to relate this lectin to analogous proteins of *Streptomyces*. Homologous sequences were identified as N-terminally truncated hypothetical proteins in the genomes of *S. lavendulae* and *S.* sp. MG1. The SHA homologue polysaccharide deacetylase from *S. lavendulae* (PDSL) was expressed in *E. coli*, and purified from inclusion bodies. Close comparison of peptides derived from SHA and PDSL resulted in the identification of a single amino acid difference in SHA out of 131 amino acids. PDSL-A108E transiently expressed in HEK293S cells showed carbohydrate-binding activity, which supported that PDSL-A108E is virtually identical with SHA. Comparison of SHA to genome-encoded hypothetical proteins revealed intriguingly that the SHA-homologous domains are found in the C-terminal domains of all hypothetical homologues so far identified. The closest homologues in *S. lavendulae* and *sp*. Mg1 contain 68 and 74 extra amino acids, respectively, at their N-terminal domains. SHA homologues contain five conserved tryptophan residues, four of which are located in three Clostridial hydrophobic with conserved W (ChW) domains, providing the unique opportunity for studying the role of ChW domains for carbohydrate binding. The fact that genomically encoded SHA-homologues were not expressed by *S. lavendulae* and *S. sp*. Mg1 emphasizes the need for regaining a SHA-producing strain to determine how SHA is expressed and secreted.

(103) Human fucosyltransferase FUT5: Crystal structure and Acceptor specificity

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The FUT gene family encodes a group of proteins that shows a complex tissue and cell type specific expression pattern. Fucosyltransferases (FUTs) are a family of glycosyltransferases that belong to the GT10 family of carbohydrate modifying enzymes. FUTs catalyze the final step in the synthesis of a range of glycoconjugates with different immunological and functional properties including blood group antigens and selectin ligands. Interestingly, the acceptor substrate specificity of different forms of the human FUTs with highly homologous amino acid sequences can differ significantly. FUTs display different but sometimes overlapping enzymatic properties enabling differentiation between the various enzymatic activities by assaying their substrate specificity patterns.

In general, all human GT10 fucosyltransferases (FUT3-7 and FUT9-11) synthesize α1,3-fucosyl linkages to GlcNAc residues in Gal-GlcNAc (LacNAc) sequences using GDP-fucose as the sugar donor. Several members of the GT10 family have unique specificities for using the GlcNAc residue in Gal-β1,4-GlcNAc (type 2 LacNAc) acceptors with modifications to the Gal residue, including sialylated and H-type fucosylated structures. In addition, FUT3 and FUT5 also have the ability to transfer fucose to GlcNAc residues in type 1 LacNAc sequences (Gal-β1,3-GlcNAc) in an β1,4- linkage, as well as acting on type 2 LacNAc chains (± modifications to the Gal residue). Thus, FUT9 can synthesize α1,3-Fuc linkages on both type 2 and H-type 2 acceptors (but not sialylated type 2 sequences), while FUT5 is more promiscuous and can add fucose residues to unmodified, sialylated, or H-type type 1 or type 2 acceptors.

Here we present the first crystal structure of the human α1,3-fucosyltransferase, FUT5, which was solved as a GDP complex adopting a metal-ion independent glycosyltransferases-B fold with two domains separated by an acceptor-binding pocket. Potential residues which may impact the substrate

specificity and catalysis were revealed on the basis of its structure. Based on this study we generated the series of catalytic mutants of FUT5 to assay the acceptor specificity. Kinetic assays for each mutant and co-crystallization of FUT5 with acceptor substrate is underway. This poster presents models for the catalytic mechanism for the FUT5 and comparison of its specificity towards different acceptor substrates.

(104) The roles played by the other half of a glycoconjugate: contributions of scaffolds to lectin-glycoconjugate interactions

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A glycoconjugate, natural or synthetic, is composed of two structural entities: the glycan epitope(s) and a scaffold. Proteins, peptides, lipids, and synthetic structures serve as scaffolds of glycoconjugates. Biological activities of glycoconjugates are attributed to the attached glycans, while the seemingly inert inner scaffolds are considered to play a passive role. Scaffolds are primarily viewed as physical supports to the attached glycan epitopes. Our current study, however, shows that scaffolds actively contribute to lectin-glycoconjugate interactions by influencing the binding thermodynamics. In addition, lectin-mediated cross-linking properties of glycoconjugates can also be modulated by their scaffolds. We found that when a free glycan was attached to a scaffold, its lectin binding entropy became more positive. The level of positive entropic gain was dependent on the nature of the scaffolds tested. For example, protein scaffolds of glycoproteins were found to generate more positive entropy of binding than synthetic scaffolds. Some scaffolds were found to have limiting effects on the affinity of glycoconjugates. We also observed that glycoconjugates with a similar affinity and an identical valence demonstrated different kinetics of lattice formation with lectins, when their scaffold structures were different. Our findings support the view that scaffolds of glycoconjugates (i) control the entropy of binding during lectin recognition and (ii) help diversify the cross-linking properties of glycoconjugates. Valence and affinity are the principal determinants of lectin-glycoconjugate interactions. The present study showed that the influence of scaffolds on lectin–glycoconjugate interaction could be independent of the valence and affinity of the glycoconjugates involved. In addition to affinity and valence, the scaffold of a ligand could be an additional factor that controls the outcome of lectin-glycoconjugate interaction. Therefore, lectin binding properties of glycoconjugates could be altered by fine-tuning their scaffolds. Our data also suggest that glycoconjugates with comparable affinities and similar valence are functionally unique when the structures of their scaffolds are different [1].

Reference

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(105) Comparative analysis of N-glycans in skeletal muscle cells and its exercise condition

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Diabetes is one of the typical disease to cause various complications such as neuropathy, retinopathy and nephropathy. It has been thought that the cause is long term hyperglycemic condition. Disturbance of motor function, and muscular atrophy are also induced by long term hyperglycemic condition, and these symptoms are considered to be some effect on the muscle cells. In fact, exercise therapy is adopted to diabetes treatment. However, it has not been revealed about the details. Skeletal muscle is important organ in metabolism and exercise, and it is the largest organ in the body. In addition, most of molecules in the skeletal muscle have modified with glycosylation. Therefore, we focused on the structure of N-glycans of skeletal muscle cells in hyperglycemic condition. In our previous study, we analyzed N-glycan structures of two types conditions of normal blood sugar (Low Glucose Condition Cells; LGCC) and hyperglycemic (High Glucose Condition Cells; HGCC) in the differentiated C2C12 cells. The result of the comparative structural analysis, it was confirmed that the expression level of the specific N-glycan structures were changed. We suggested that another N-glycan synthetic pathway is activated within the cells at the long term hyperglycemic condition. In this study, we analyzed the correlation of the N-glycans structure and those expression at exercise condition in skeletal muscle cells. The differentiated C2C12 cells (myotube form) was treated by electrical pulse stimulation (EPS) which was the pseudo-exercise condition. After the cultivation at above condition, all N-glycans were liberated from C2C12 cells by hydrazinolysis and fluorescent-labeled by pyridylamination. The structure and amount of the pyridylaminated N-glycans were determined by HPLCs and MALDI-TOF-MS. The result of comparative structural analysis of N-glycans, the relative amount of N-glycans was decreased by EPS treatment as compared with those of no EPS treatment.

(106) Evolutionary analysis of UDP-GlcNAc binding site in O-GlcNAc transferase using the modify evolutionary trace method

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O-GlcNAcylation is an essential post-translational modification in metazoa. This modification is catalyzed by *O*-GlcNAc transferase (OGT). OGT activity has been reported in Metazoa and Viridiplantae, but not in Monera.

We previously reported that the amino acids in the vicinity of UDP-GlcNAc binding site in OGT have not been conserved by using the evolutionary trace method, which predicts protein functional sites by identifying evolutionarily conserved amino acids and mapping them on 3D structures. However, evolutionary trace method fundamentally ranks amino acid depending on the degree of its conservation without consideration for physicochemical property of amino acids. Consequently there is a high possibility of producing a false-negative resultby this method. In the present study, we analyzed amino acid conservation in consideration of physicochemical property and trend in conserved amino acid.

We conducted PSI-BLAST search with human OGT (Swiss-Prot accession number O15294) as a query sequence to search its homologous sequences from NCBI. The resulting protein sequences were aligned by Clustal. Moreover we searched amino acid conservation in consideration with trends in conservation status and amino acid property, and mapped them onto 3D structures. We especially focused on amino acids within 3.5Å from UDP-GlcNAc using a human OGT crystal structure (PDBID: 4gz5).

As a result of the analysis, we found many sites showing more than 90% conservation among amino acids around the uracil and ribose and some sites showing 80-89% conservation around pyrophosphoric acid. Amino acid site showing less than 70% conservation are present around GlcNAc. Many sites showing more than 90% conservation seem to tend to be replaced with different amino acid in some bacterial species. Moreover, sites showing 80-89% conservation were not conserved in many bacteria.

Consequently, amino acids around uracil and ribose are inferred to have been conserved before occurrence of eukaryotes. Because amino acids around pyrophosphoric acid are specific to metazoa and Viridiplantae, these sites are presumed to be important in the binding of UDP-GlcNAc. The sites around GlcNAc do not trend to conserve in Bacteria. Accordingly these sites are presumed to have changed to be able to bind to UDP-GlcNAc in eukaryotes.

(107) Structure and biosynthesis of complex N-glycan cores and antennae in nematodes

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There are thousands of nematode species, some of which are parasites such as Ascaris suum, Trichuris suis or Haemonchus contortus; others such as the popular model organism, Caenorhabditis elegans, are free-living. Although all are animals, they lack some of the characteristics of glycosylation found in vertebrates, but their 'foreign' nature is relevant to the interactions of nematode parasites with their hosts. The presence of complex modifications of the chitobiose core region of nematode N-glycans and of phosphorylcholine on the glycan antennae (also in series) are typical, but the details are species-specific. Whereas Caenorhabditis N-glycans can contain up to four fucose residues, this number is reduced in parasitic species. Also galactosylation, at unusual positions, is a recurrent theme. Of the three core fucosyltransferases (FUT-1, FUT-6 and FUT-8) and one fucose-modifying galactosyltransferase (GALT-1) known, unusual and unexpected enzyme specificities have been revealed by studying both enzyme specificities and the effect on the glycome of deleting the relevant genes; even the processing hexosaminidases (HEX-2 and HEX-3) assumed to reside in the Golgi apparatus of nematodes are only distantly related to their 'cousins' in other invertebrates which are responsible for formation of paucimannosidic glycans. Thereby, glycomics, genetics and biochemistry are not only important tools for understanding nematode glycan biosynthesis, but open the way to identifying carbohydrate motifs which play roles in the interactions of these organisms with their hosts.

(108) Role of the oxygen-dependent Skp1 glycan in Skp1 organization in Dictyostelium

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Skp1 is a highly conserved adaptor that links Cullin-1 to F-box proteins in SCF (Skp1-Cullin1-F-box) E3 ubiquitin ligases. In the social amoeba *Dictyostelium discoideum* and other protists including the parasite *Toxoplasma gondii,* Skp1 is regulated by a novel pentasaccharide attached to a hydroxyproline near its C-terminal F-box binding domain. Skp1 prolyl hydroxylation is involved in O_2-dependent developmental regulation, and genetic studies show that subsequent glycosylation is required for full realization of the hydroxylation effect. The glycan promotes increased order of the Skp1 polypeptide, and association of Skp1 with several F-box proteins. NMR studies were employed to define the non-reducing terminal glycosidic linkage and investigate how the glycan may promote Skp1 polypeptide organization and association with

F-box proteins. Analysis of glycopeptides from Pronase-digested recombinant Skp1 containing αGal residues labeled at their C1-positions with ^{13}C revealed that both αGal residues are 3-linked to their underlying sugars, showing the overall structure to be Galα1,3Galα1,3Fucα1,2Galβ1,3GlcNAcα1-4Hyp(Skp1). Analysis of native Skp1 in which the (peripheral) αGal residues were U-^{13}C-labeled confirmed their linkages in the intact protein. Rotational correlation values for this and a version of Skp1 in which the core αGlcNAc was 13C-labeled revealed coordinated yet relatively free movement of the individual sugars relative to a distal ordered region of ^{15}N-labeled Skp1. Nuclear Overhauser signals indicated a preferred spatial relationship between most sugars. Molecular dynamics simulations confirmed that the glycan has sufficient internal organization to mediate a second-site interaction with F-box proteins or with itself, and that flexibility needed for orientation could originate from the linkage to and puckering of hydroxyproline. NMR analysis of $^{15}N/^{13}C$-labeled Skp1 revealed that the C-terminal region of Skp1, which harbors the glycosylation site and associates with F-box domains, becomes more organized when glycosylated, consistent with results from circular dichroism and small angle X-ray scattering. Molecular dynamics simulations support this interpretation but suggest altered conformational trajectories of the two terminal helices consistent with the idea of conformational selection by F-box domains to achieve the stereotyped structures observed in crystallographic studies of the complexes.

(109) Protein O-mannosylation is required for normal sensory feedback and coordinated muscle contractions in Drosophila

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Protein O-mannosylation (POM) is known to significantly affect mammalian muscular and neural development. Defects in the biosynthesis pathway of O-mannosyl glycans lead to congenital neuromuscular disorders and targeted deletion of protein O-mannosyltransferases (POMTs) cause lethality in mice. Mammalian POMT family consists of POMT1 and POMT2, which are coexpressed in the endoplasmic reticulum and function as a heterocomplex. *Drosophila* has orthologues of POMT1 and POMT2, known as RT (Rotated Abdomen) and TW (Twisted), respectively. Defects in these enzymes cause a prominent phenotype of clockwise rotation of abdominal segments in adult flies and larval muscle abnormalities. A known target of O-mannosylation in *Drosophila* and mammals is Dystroglycan, whose hypoglycosylation leads to muscle degeneration and failure of neuronal migration. Despite numerous studies of POM functions, the molecular and genetic mechanisms responsible for causing these defects still remain elusive. To unravel these pathogenic mechanisms, we studied the function of *POMT* genes in *Drosophila* embryo. We found that the late *Drosophila* embryo shows a prominent phenotype associated with abnormal coordination of muscle contractions. We hypothesized that this phenotype might shed light on the pathogenic mechanism common for different developmental stages. To test this hypothesis, we employed the UAS-GAL4 system and rescue strategy to investigate the requirement for POM in muscles and neurons. Interestingly, we found that the phenotypes could be rescued significantly by expressing POMT enzymes in neurons only, including multi-dendritic sensory neurons, but not in muscles. This suggested that POMTs have a specific role in these neurons and that down-regulation of POM causes an abnormal sensory feedback and affects the pattern of muscle contractions, thus underlying the pathogenic mechanism of POMT phenotypes. We further investigated the role of POMTs in neurons by analyzing their axonal projections in the ventral ganglion. We found significant abnormalities in the morphology of axonal tracts, supporting our hypothesis that POMTs are required for proper function of sensory neurons and hence, for coordinating the pattern of muscle contractions. The molecular targets of POM in this pathway remain to be determined and our current research focuses on that aspect.

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(110) Nutrient-driven O-GlcNAc cycling impacts Neurodevelopmental Timing and Metabolism

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Nutrient-driven *O*-GlcNAcylation is strikingly abundant in the brain and has been linked to signaling and development. We selectively targeted the *O*-GlcNAcase gene in the mouse brain to define the role of *O*-GlcNAc cycling in the central nervous system. The brain-specific knockout animals exhibited dramatically increased brain *O*-GlcNAc levels and pleiotropic phenotypes including growth defects, obesity and metabolic dysregulation. Anatomical defects in the *Oga* knockout included delayed pituitary differentiation and neurogenesis. The molecular basis for these defects included transcriptional changes accompanying embryonic stem cell (ES)-derived neural differentiation *in vitro*. In the *O*-GlcNAcase knockout ES cells, pronounced changes in expression of pluripotency markers were observed including changes in *Oct-4*, *Nanog*, and *Otx-2*, a homeobox transcription factor with an established role in the maintenance of primed pluripotency, neurogenesis and pituitary development. We obtained both *in vivo* and *in vitro* evidence that OTX2 is both precociously expressed and stabilized by the loss of *O*-GlcNAcase function. These findings are consistent with an OTX2-dependent increase in proliferation and decreased neuronal and pituitary differentiation accompanying the developmental delay in the *Oga* brain KO mice. These findings link the O-GlcNAc modification to the process of mammalian neurogenesis and highlight the role of

this nutrient-sensing pathway in developmental plasticity and metabolic homeostasis.

(111) Subcellular expression of core fucosylated glycoproteins in postmortem human cortex: preliminary evidence for targeted glycoproteomic evaluation of schizophrenia brain

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One of the most serious psychotic illnesses, schizophrenia, is a neurodevelopmental psychiatric disorder resulting from a variable constellation of heritable and environmental factors. Approximately 1% of the population is afflicted by this disorder, but an unfortunate dearth of tolerable and effective treatment options makes the identification of novel therapeutic targets a research imperative. Disrupted patterns of glycosylation have been identified in schizophrenia brain including altered N-glycosylation of neurotransmitter receptor and transporter subunits, as well as altered gene and protein expression of several key glycosylation enzymes. FUT8 is a non-redundant α-1,6-fucosyltransferase that catalyzes the core fucosylation of N-glycans. Our lab has found alterations of FUT8 transcript levels and decreased FUT8 protein expression in schizophrenia superior temporal gyrus (STG). Additionally, Aleuria aurantia lectin (AAL), which recognizes the FUT8 reaction product—core fucose—demonstrates reduced binding to glycoproteins in schizophrenia STG. To identify core fucosylated glycoproteins with translational relevance, we performed lectin affinity capture from samples of total brain homogenate and nuclei- and synapse-enriched biochemical fractions using biotinylated AAL and streptavidin-coated magnetic beads, then subjected fucosylprotein-enriched samples to nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) in a data-independent acquisition experiment. A total of 202 distinct fucosylproteins were identified, with 91 proteins enriched in the synaptic fraction and 76 in the nuclear fraction. InterPro protein domain and feature analyses reflected enrichment of proteins containing kinase domains in the synaptic fraction and proteins containing ATPase or immunoglobulin domains in the nuclear fraction, while molecular function analyses revealed variable enrichment of proteins involved in catalytic processes, molecule binding, signaling pathways, and structural components between subcellular fractions. Previous evidence of involvement in nervous system development or neurological disorders has been reported for 106 of the identified proteins and specific involvement in schizophrenia pathophysiology has been reported for 18 fucosylproteins. Given these findings, quantification of the identified fucosylproteins and assessments of their core fucosylation status in schizophrenia brain will likely suggest promising candidate mechanisms for pharmacologic intervention.

(112) Polysialic acid synthesis by ST8SIA2 is essential for cortical interneuron development

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The neural cell adhesion molecule NCAM and its modification with polysialic acid (polySia) are major determinants of brain development. Polysialylation of NCAM is implemented by the polysialyltransferases (polySTs) ST8SIA2 and ST8SIA4. Dysregulation of polySia-NCAM is implicated in psychiatric disorders such as schizophrenia. Previously, we described reduced interneuron densities in the prefrontal cortex of ST8SIA2- or ST8SIA4-negative mice and demonstrated that acute enzymatic removal of polySia in organotypic slice cultures causes impaired entry of interneurons into the embryonic cortex as well as slower migration within the cortical environment (Kröcher *et al.* 2014, Development 141:3022). However, the spatial-temporal impact of the two polySTs on the migration process remained unresolved. Here we characterize altered interneuron migration by live cell imaging in organotypic slice cultures of ST8SIA2-deficient embryos. To distinguish between a cell-autonomous effect of polySia produced by ST8SIA2 in migratory interneurons or in the cortical environment, the migration of GFP-labelled interneurons from the medial ganglionic eminence (MGE) into the cortex was studied in MGE- and cortex-explants obtained from wildtype or ST8SIA2-deficient embryos and co-cultured in different combinations. A potential link between cell-autonomously altered polySia and reduced cortical interneuron densities was assessed by analyzing mice with a conditional knockout of ST8SIA2 in MGE-derived interneurons (Lhx6-Cre) and in the cortex (Emx1-Cre). The results point towards a differential contribution of ST8SIA2 to cortical interneuron migration and the establishment of interneuron densities in the mouse cortex.

(113) COG Deficiency Drastically Alters Mucin-Type Glycosylation on Alpha-Dystroglycan Increasing its Proteolytic Susceptibility

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Deficiency in subunits of the conserved oligomeric Golgi (COG) complex results in pleiotropic effects on protein and lipid glycosylation and cause human disease. Insight regarding the specific glycoproteins affected and the functional consequences of this defective glycosylation, however, are lacking. We adopted a chemical glycobiology approach to identify cell surface glycoproteins most sensitive to the

altered Golgi function in COG-deficient CHO cells. Our results show a profound loss of sialylation following ManNAz labeling in Cog1-deficient ldlB cells but an unexpected increase in the labeling of specific glycoproteins using GalNAz. One of the heavily GalNAz-labeled glycoproteins was identified as alpha-dystroglycan (α-DG) by proteomic analysis on the basis of its preferential loss from the cell surface following V. cholera neuraminidase treatment. Western blot analysis showed that α-DG in ldlB cells has significantly lower molecular weight (~60KDa) compared to that of α-DG in WT CHO cells (~130 KDa). This decrease was largely due to simpler elaboration of the O-glycans on α-DG in ldlB cells as determined by glycomic analysis of O-glycans and enzymatic labeling (SEEL). We further showed that the underglycosylated α-DGmolecules on ldlB cells exhibit a higher rate of turnover and are more vulnerable to both bacterial proteases (including a mucinase enzyme contaminant found in the V. cholera neuraminidase preparations) and to human metalloproteinases. Similar susceptibility of α-DG was noted in Cog2-deficient ldlC CHO cells but not in sialylation-deficient Lec2 cells, suggesting that the protective effect of the glycan is mediated by non-sialic acid sugars. Addition of terminal glycosylation onto cell surface α-DG using SEEL partially restored protease resistance in ldlB cells. In summary, we demonstrate profound effects on the mucin-type glycosylation of α-DG in a COG-deficient background, and show that altered elaboration of these glycans results in increased turnover and greater susceptibility to proteases. Our discovery highlights a novel function for the mucin-type O-glycans found on α-DG that is distinct from the O-mannose initiated structures associated with congenital muscular dystrophies. This work also supports the growing role of mucin-type glycans as regulators of extracellular proteolysis and suggests a mechanism whereby COG deficiency might cause tissue-specific pathology in human patients.

(114) Glycans on human undifferentiated pluripotent stem cells revealed by using newly generated monoclonal antibodies, R-10G and R-17F

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When human iPS cells were discovered, 10 years ago, we already had marker antibodies for hiPS cells such as SSEA-3/4 and TRA-1-60/81, which recognize globosides and keratan sulfate, respectively. However these mAbs recognized embryonal carcinoma (EC) cells as well as hiPS/ES cells. On this background, we generate antibodies, R-10G and R-17F, specific to hiPS/ES cells by immunizing mice with human iPS cells, followed by differential screening of hybridomas (1, 2).

Cell surface distribution of R-10G epitope on hiPS cells was distinct from those of TRA-1-60/81, suggesting that a single colony of undifferentiated hiPS cells consists of cell subtypes with different glycans. The R-10G carrier protein isolated from hiPS cell lysates with an affinity column of R-10G was identified as podocalyxin by LC/MS/MS analysis. The R-10G epitope was a type of keratan sulfate with some unique properties. Upon ELISA test on avidin-coated plates using biotinylated derivatives of a series of *N*-acetyllactosamine tetrasaccharides (keratan sulfates (KSs)), the minimum epitope structure was shown to be Galβ1−4GlcNAc(6S)β1−3Galβ1−4GlcNAc(6S)β1. Removal of sulfate from GlcNAc(6S) or addition of sulfate to Gal reduced the binding activity almost completely.

The R-17F antibody (IgG1 subtype), which bound to almost every undifferentiated pluripotent stem cell effectively, exhibited a strong cytotoxic effect on hiPS/ES cells. The cytotoxic effect was augmented markedly upon the addition of the secondary antibody (goat anti-mouse IgG1 antibody). R-17F may be beneficial for safer regenerative medicine by eliminating residual undifferentiated hiPS cells in hiPS-derived regenerative tissues, which are considered to be a strong risk factor for carcinogenesis.The R-17F antigen was initially isolated from a total lipid extract of hiPS cells and identified as LNFP I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc). But later, it was found that R-17F epitope was expressed also on podocalyxin (3).

TRA-1-60, which is probably a most frequently used stem cell marker antibody, is also expressed on podocalyxin. ELISA test with a series of *N*-acetyllactosamine tetrasaccharides as described above indicated that the TRA-1-60 epitope is distinct from that of R-10G.

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(115) Structural and biochemical analyses suggest that O-fucose and O-glucose glycans modulate protein folding and flexibility of EGF repeats

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O-Fucose and *O*-glucose glycans are attached to the epidermal growth factor-like (EGF) repeats in the extracellular domain of Notch and critically regulate Notch receptor activation. *O*-Fucose glycans are stably associated with underlying amino acids of Notch1 EGF repeat 12, functioning as "surrogate amino acids" at the ligand-binding interface. Genetic and cell biological analyses of the glycosyltransferases responsible for the biosynthesis of *O*-glucose glycans indicated that they are also essential for Notch activation, while xylosyl-extension of *O*-glucose negatively regulates Notch activation. The molecular mechanism of this

regulation is not understood. Our recent structural analysis of human factor IX EGF repeat modified with an *O*-glucose disaccharide, Xylα1-3Glcβ1-*O*-Ser, co-crystallized with xyloside xylosyltransferase 1 showed that the EGF portion of the acceptor was highly distorted, but still retained three disulfide bonds when bound to the enzyme, which revealed the flexible nature of an EGF repeat.

Here we solved the crystal structure of human factor IX EGF repeat modified with an *O*-glucose trisaccharide, Xylα1-3Xylα1-3Glcβ1-*O*-Ser. The *O*-glucose trisaccharide laid on the surface of the EGF repeat through the intramolecular interactions with underlying amino acids. In order to examine the structural effect of *O*-glucose glycans on the stability of the EGF repeat, we developed a simple, HPLC-based, quantitative unfolding assay. Using this assay, we tested the stability of several glycoforms of human factor IX EGF repeat (unmodified, modified with *O*-glucose glycans, *O*-fucose glycans, or both). Both *O*-fucose and *O*-glucose monosaccharide stabilized the EGF repeat in an additive manner. Unexpectedly, addition of the first xylose destabilized the structure whereas the second xylose reversed the effect. These results suggest that *O*-fucose and *O*-glucose glycans modulate protein stability of a single EGF repeat through intramolecular interactions independently.

Ligand binding to Notch followed by ligand endocytosis into signal sending cells generates a pulling-force that is required for Notch receptor activation in signal receiving cells. Thus, modulation of the flexibility of individual EGF repeats by *O*-glycans may play an important role in regulating the activation of Notch. This work was supported by NIH grants to R.S.H (GM061126) and to H.L. (AG029979).

(116) Drosophila NGLY1 homolog is required for embryonic midgut development

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The *N*-glycanase 1 gene (*NGLY1*) encodes an evolutionarily conserved deglycosylating enzyme that acts on *N*-glycoproteins. Mutations in human *NGLY1* cause a rare congenital disorder with severe developmental delay, multifocal epilepsy, involuntary movements, abnormal liver function, gastrointestinal dysfunction, small hands/feet and absent tears. In this work, using mutant and cross-species transgenic analyses in *Drosophila melanogaster* combined with yeast experiments, we show that *Drosophila pngl* (PNGase-like) exhibits a high degree of functional conversation with human *NGLY1*. Loss of *pngl* results in a severe developmental delay and semi-lethality, with rare adult escapers (<1%) that exhibit a high frequency of early death and sterility. These phenotypes can be rescued by transgenic expression of wild-type *NGLY1* but not by a mutant *NGLY1* identified in a patient. Loss of *pngl* results in structural and functional defects in the larval midgut during development. Tissue-specific knock-down and rescue experiments indicate that the function of *Drosophilapngl* is primarily required in the mesoderm to promote Dpp signaling. Together, these results validate fly as a model to understand the function of NGLY1 and may provide a framework for understanding the pathophysiology of NGLY1 deficiency in human patients. I will present our recent findings on the mechanisms by which Pngl regulates Dpp signaling in a tissue-specific manner.

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(117) A new congenital disorder of glycosylation is due to mutations in Fucokinase

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Mutations affecting the glycosylation machinery cause an expanding spectrum of genetic disorders termed Congenital Disorders of Glycosylation (CDG). Most of the 120 distinct CDGs effect nearly ever organ system.

We identified an individual showing early-onset, intractable seizures, severe encephalopathy, unusual metabolic features, and recurrent infections. Whole exome sequencing identified two rare heterozygous mutations (p.Ser223Pro and p.Arg683Cys) in Fucokinase (FUK).

Fucokinase generates fucose-1 phosphate and ultimately GDP-fucose, the donor substrate for fucosyltransferases. This "*salvage*" pathway is thought to make only a minor contribution to total GDP-Fucose. The major "*de novo*" pathway involves conversion of GDP-mannose to GDP-Fucose.

We confirmed the pathogenicity of these variants in patient fibroblasts by showing a nearly threefold lower incorporation of [H^3-5,6]-Fucose into glycoproteins compared to three unrelated controls. The levels of GDP-[H^3] fucose and -[H^3] Fucose-1p were also reduced an average of 2.5-fold. Importantly, lentiviral transduction of wild-type *FUK* completely restored all three biochemical defects. Furthermore, western blot showed a complete loss of FUK protein, further supporting FUK deficiency.

Based on the patient's pathology, our results suggest that the *de novo* GDP-Fucose biosynthesis is insufficient in some cells and that the salvage pathway is critical. The complete absence of FUK protein along with significant residual incorporation of [H^3-5,6]-Fucose into glycoproteins suggests another FUK-independent salvage pathway in humans.

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(118) Identification and expression analysis of zebrafish polypeptide a-N-acetylgalactosaminyltransferase genes during the embryonic development

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Mucin-type O-glycosylation is one of the major post-translational modifications of membrane and secreted proteins. The glycosylation is initiated by a polypeptide α-N-acetylgalactosaminyltransferase (GalNAc-T) that catalyzes the addition of GalNAc to Ser/Thr residues in proteins. Thus, GalNAc-T is very important in that it determines the numbers and positions of mucin-type O-glycans. The human GalNAc-T family encoded by *GALNT* genes consists of a large gene family with 20 isozymes. This indicates that O-glycosylation in the tissue is determined by their selective expressions and cooperative actions in the cell.

Recent studies demonstrate that the *GALNT* isozymes play important roles in several physiologically important events, such as control of proteolytic cleavages of proteins, and in diseases such as cancer, and lipid metabolism disorder. Despite these reports on O-glycan functions, in most cases GalNAc-T isozymes that are responsible for glycosylation of target proteins remain to be identified.

In this report, in an attempt to obtain information for clarifying physiological roles of *galnt* genes, we identified and characterized zebrafish *galnt* genes, and determined their spatial and temporal expressions during zebrafish early development. We found that zebrafish have 18 *galnt* genes and the isozymes have conserved domain/motif organizations characteristic of *GALNT* genes. RT-PCR and whole mount *in situ* hybridization (WISH) demonstrated that the zebrafish isozymes have overlapping, but characteristic expression patterns during the embryonic development as seen in other organisms. The information on the isozyme expression patterns, when used together with the gene knockdown/knockout technology, will provide functional analysis of the isozymes and endogenous substrates in zebrafish. We are generating zebrafish mutants lacking a galnt gene using methods such as TALEN- and CRISPR/Cas-systems.

(119) Generation of mutant zebrafish that lack multiple vertebrate-specific polypeptide N-acetylgalactosaminyltransferases

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Polypeptide N-acetylgalactosaminyltransferases (GalNAc-Ts) that catalyze the initial reaction of mucin-type glycan biosynthesis are biochemically important enzymes because they determine the number and positions of the glycans in proteins. GalNAc-Ts consist of a large gene family with 20 isozymes in humans, and their biological functions largely remain unclear. We have been analyzing their functions with focus on a galnt subfamily, so-called Y-subfamily, that occurs specifically in vertebrates.

We previously isolated all GalNAc-T genes including Y-subfamily genes, *galnt8, galnt9, galnt17, galnt18a* and *galnt18b*, from zebrafish and demonstrated their expression patterns in the embryos (1). We found that the isozymes with high expression in mammalian brains were also highly expressed in the zebrafish brain. Recently we have established mutant zebrafish lines of Y-subfamily, *galnt9* and *galnt18a* with TALEN methods and found that they did not show significant phenotypic alterations during the early development, suggesting the possible redundant functions of family isozymes in the same tissues. To overcome the problems that are sometimes found in molecules with a large gene family, multiple knockout/knockdown of genes expressed in the same cells/tissues may be required.

We are now establishing mutant lines for additional Y-subfamily isozymes, *galnt17* and *galnt18b*, using a CRISPR/Cas9 method. However, it usually takes more than a half year to establish mutant lines by editing genomes and following conventional family inbreeding, and hence it is laborious to obtain multiple knockout organisms by the usual genome editing. An improved CRISPR/Cas9 method was recently reported in which biallelic mutations in F0 generation are produced with high efficiency by introducing a zebrafish codon-optimized Cas9 mRNA or purified Cas9 protein together with single guide RNAs (sgRNAs). We are applying the improved CRISPR/Cas9 method and the morpholino antisense technology to Y-subfamily mutant lines in order to obtain the mutants with multiple Y-subfamily genes knocked-out and/or -down. Their phenotypic alterations will be analyzed to investigate the developmental roles of GalNAc-T Y-subfamily *in vivo*.

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(120) N-Glycosylation Changes in the Human Aortic Valve Structure during Development and Disease by Imaging Mass Spectrometry

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Introduction: Changes in polysaccharide and proteoglycan distribution are a hallmark of heart valve development and disease. However, the complex carbohydrate content of the

heart valve remains virtually uncharacterized at the molecular level. Our proteomics data on healthy & diseased human aortic valve (AV) showedsignificant enrichment offunctions of N-glycan processing, synthesis and congenital disorders of glycosylation. This led us to undertake an investigation on human aortic valve tissue directed at understanding N-glycan tissue patterning and alterations in healthy development and due to congenital aortic valve stenosis.

Methods: Imaging mass spectrometry was used to map distinct N-glycan features on de-identified aortic valve tissue. Tissue was heated, dewaxed, antigen retrieved in citraconic buffer. PNGase F was sprayed onto formalin fixed paraffin embedded (FFPE) tissue sections (5-6 μm) to release N-glycans from proteins without delocalization using a robotic sprayer (TM-Sprayer, HDXImaging Technologies). MALDI Fourier transform ion cyclotron resonance mass spectrometry (7 T Solarix, Bruker Daltonics) was used to sample tissue at a 40 μm step size. Image data were visualized by fleximaging (v4.1 Bruker Daltonics) and analyzed by SCiLS software (v2016, SCiLS, GmbH).

Results: From a growing cohort, distinct N-glycans are spatially restricted and regulated within the human AV structure. In healthy AV, N-glycan expression patterns are segregated according to AV tissue features. In CAVS, distinct N-glycans were increased in regions of pathological ECM mixing. Statistical filtering by Wilcoxon Rank Sum p-value ≤1.0E-3 and area under the receiver operating curve ≥0.7 suggested expression level changes in 27% of N-glycan (30/110) when comparing between healthy AV ages 0-17 years and the mature adult tissues. Early structural motif analysis indicates that in the healthy aortic valve, fucosylation decreases during maturation while N-glycan complexity increases. In comparison of age-matched healthy AV to CAVS tissues, N-glycan structural motifs increase in complexity, including sialylated species and, putatively, multiply fucosylated species. The study is showing that distinct N-glycans play an important, but currently unknown role in ECM organization and regulation of the AV structure. Work is underway to define the cellular processes associated with the observed N-glycan changes and further characterize observed sialic acid linkages through on-tissue chemistries.

(121) A Uniquely Human Evolutionary Change in ST8Sia-II Impacts Enzyme Stability and Polysialic Acid Function

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Through investigation of genetic changes that occurred after the divergence of humans from our closest living hominid relatives, we have identified a uniquely human evolutionary change in the amino acid sequence of ST8Sia-II, one of the two highly conserved sialyltransferases that are responsible for the synthesis of polysialic acid (PolySia). This evolutionary change, N308K, is intriguing because this is the only amino acid that differs between the human ST8Sia-II enzyme and that of non-human hominids. While the ancestral N308 is conserved in all non-human mammals, and even in distantly related species through *Xenopus laevis*, K308 is fixed in all human populations and is present in the Neanderthal and Denisovan genomes, suggesting selection for this change during early human brain evolution. Despite hundreds of millions of years of conservation at this basic residue, ST8Sia-II retains the ability to polysialylate human NCAM when K308 is mutated to an alanine, suggesting that conservation at this site is involved in some other aspect of polysialylation. Proper spatial and temporal regulation of polySia synthesis is critical in neural development, plasticity, and regeneration, as well as implicated in psychiatric and neurodegenerative disease. Thus, the uniquely human change may be involved in some of the unique aspects of the human brain such as advanced cognitive abilities, developmental delay, and susceptibility to psychiatric and neurodegenerative diseases. We have identified a reduction in the stability of the enzyme as a result of the human mutation, implying that it may play a role in increased turnover and regulation of the transferase. We have also identified a marked difference in binding affinity of BDNF and FGF2 between polySia-NCAM molecules synthesized by the human and chimpanzee form of the enzyme. This preliminary data indicates that the uniquely human change in ST8Sia-II has functional consequences upon which evolutionary selection could have acted. Further experiments are underway to characterize the impact that this change has upon polySia-dependent processes in human neural cells. Finally, we have produced a mouse harboring the human-like *St8sia2* N308K change and are currently characterizing behavioral, aging, and brain-widephenotypes.

(122) Analysis of Changes in Glycosylation as Pluripotent Human Stem Cells Differentiate into Separate Germ Cell Lineages

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We are studying human embryonic stem cell (hESC) development as a model for changes in glycan-related gene transcripts and glycan structural changes that occur during cellular differentiation. Previously, we used a high-throughput qRT-PCR platform to analyze transcripts for a

collection of ~900 glycan-related genes in populations of pluripotent and differentiated human stem cells. We are currently expanding our analysis to include the entire transcriptome using RNA-Seq to examine transcript changes that occur during stem cell development, including those that may interact with or regulate glycan-related gene expression. Prior RNA-Seq efforts optimized strategies for aligning sequencing reads and validated the repeatability of technical replicate runs ($R^2 > 98\%$). We have now optimized strategies for differential transcript expression and validated these analyses through comparison of our RNA-Seq and qRT-PCR data sets. Biological replicate analysis was incorporated into our qRT-PCR workflow to provide statistics on biological variation of transcript abundance while reducing the costs of replicate analysis by RNA-Seq. We are also employing multiplexed analysis of samples for RNA-Seq through the use of barcoding to increase sample throughput at reduced cost. 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. We will present RNA-Seq 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). This set of differentiated cell lineages encompasses all three germ layers; endoderm (Liver), mesoderm (WT-1 and SM) and ecotoderm (NC), which may highlight germ layer-specific changes in glycan-related gene expression and glycan structural information. Metabolic pathways will be used to highlight changes in glycan-related transcript abundances between ES and differentiated cell lines. Transcript changes that are uniquely regulated in different stages of differentiation will be highlighted. A comparison of the recently completed N-glycan structural analysis with the transcript analysis from matched samples will be presented. (supported by NIH grant P41GM103490 to JMP).

(123) The role of Nrf1 in NGly1 deficiency

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Recently a rare inherited congenital disorder, N-Glycanase 1 (NGly1) deficiency, caused by heterozygous inactivating mutations in the *ngly1* gene has been discovered. Patients suffering from NGly1 deficiency exhibit a spectrum of symptoms, such as global developmental delay, hypotonia, seizures, peripheral neuropathy, alacrima and liver abnormalities. NGly1 is thought to function as a key component of the ER-associated degradation (ERAD) machinery. By catalyzing the de-N-glycosylation of glycoproteins it plays an important role in degradation of 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. Therefore, we wondered if the physiologically relevant role of NGly1 during human development might be more specialized than previously thought.

One hypothesis is that a single NGLy1 substrate might be dependent on de-N-glycosylation for its function and could therefore be the underlying mechanism mediating disease pathologies. Our first candidate is the transcription factor Nuclear Factor Erythroid-2 Related Factor 1 (NFE2L1, also called Nrf1) which is a member of the cap'n'collar (CNC) bZIP family. Nrf1 is a master regulator of many vital metabolic pathways, including the transactivation of antioxidant enzymes, phase 2 detoxification, and proteasomal subunit gene expression. Its activation involves retro-translocation from the ER to the cytosol, de-N-glycosylation, and partial proteolytic processing to generate the active forms of Nrf1, which translocate to the nucleus to effect gene transcription. Under conditions of normal proteostasis Nrf1 is continually targeted for proteasomal degradation. However, under conditions where proteasome activity is compromised, such as during treatment with proteasome inhibitors, deglycosylated Nrf1 accumulates and exerts its nuclear functions, including transactivation of proteasomal subunit genes (bounce back). Importantly, the phenotypes of tissue-specific knockdowns (CNS, liver, bone) overlap with some NGly1-null pathologies. On this basis, we hypothesize that impaired de-N-glycosylation of Nrf1 in the absence of NGLy1 results in an abrogated bounce back response that in turn contributes to the disease symptoms associated with NGLy1 deficiency. The first results of our investigation, which include a comprehensive comparison of different NGly1-null cell lines with their wildtype counterparts, will be discussed.

(124) Quantification of Thr vs Ser Acceptor Preferences of the ppGalNAc Transferases That Initiate Mucin Type O-Glycosylation

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Mucin type O-glycosylation is initiated by a large family of polypeptide GalNAc transferases (ppGalNAcT) that transfer the first GalNAc residue onto specific Thr or Ser residues of a substrate protein. There are 20 different ppGalNAcT isoforms in mammals and most show regulated spatial/temporal expression during development and discrete distribution in adult tissues. The sheer number of isoforms involved suggests the complex nature of substrate selection and Thr/Ser site preferences. By sequence similarity the isoforms are categorized into 9 related sub-families and according to substrate selectivity, they can be broadly divided into, naked peptide vs glyco-peptide preferring family of transferases; although recent studies from our lab show there are considerable overlaps. We have shown that the acceptor site preferences are modulated by neighboring sequence

motifs and prior glycosylation at a specific distance and orientation (N' or C' terminal). Remarkably, these preferences vary even within sequence similar subfamilies, where each isoform has unique combination of peptide/glycopeptide specificity. The overall results suggest that the addition of successive GalNAc residues onto a given substrate may be an ordered process, regulated by individual isoform preferences.

Early studies on the ppGalNAc-T1 isoform revealed that Thr acceptor residues are glycosylated at higher rates than Ser. Here we report the first study that systematically quantifies acceptor residue (Thr vs Ser) preferences between isoforms. We have determined the Thr/Ser preference ratios for about 11 isoforms by performing independent but identical in vitro reactions on randomized peptides of varying length and amino acid content, designed with either Thr or Ser acceptors in the center. The results show that all isoforms have elevated preferences for Thr residues but with different rates (Thr/Ser ratios). Interestingly the ratios are similar within a subfamily but vary between different subfamilies with values ranging from 2 to 13. The data will be incorporated into our web based tool -ISOGlyP (http://isoglyp.utep.edu) that predicts relative sites of O-glycosylation of any given protein.

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(125) Inhibition of N-glycanase1 induces autophagic clearance of protein aggregates

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Quality control of protein folding is crucial to the maintenance of cellular homeostasis. Impairment of these systems and the related degradative pathways involved in the clearance of misfolded proteins can result in severe and varied pathologies. Peptide N-glycanase (EC 3.5.1.52) is an endoglycosidase which cleaves N-linked glycans from incorrectly folded glycoproteins exported from the endoplasmic reticulum and occurs prior to degradation by the 26S proteasome and is important for the degradation of misfolded glycoproteins during ER-associated degradation. Mutations in this enzyme are responsible for the rare disorder, N-GLY1, a congenital multi-system disorder which results in a build-up of protein aggregates in the cell cytosol.

Using a pharmacological inhibitor of peptide N-glycanase, carbobenzoxy-valyl-ananyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk), we have found that inhibition of N-glycanase using 50 μM Z-VAD-fmk resulted in an increase in Thioflavin T fluorescence intensity after 48 hours which decreases to near basal levels after 72 hours. Thioflavin T is a fluorescent dye that exhibits enhanced fluorescence upon binding to β-sheets, characteristic of aggregated structures and fibril formation. Changes in characteristic ER stress markers were also observed; variation in the expression of BiP (GRP78), which is increased during the unfolded protein response, was observed to correlate with the changes in Thioflavin T fluorescence.

Using a GFP-LC3 reporter in HEK cells we found an increase in autophagy after 72 hours of peptide N-glycanase inhibition. The increase in autophagy corresponded to the decrease in Thioflavin T fluorescence and variation in BiP levels, suggesting that protein aggregates were removed by the induction of autophagy when deglycosylation is impaired. In an autophagy deficient cell line, ATG13-/- MEFs, we found inhibition of peptide N-glycanase by 50 μM Z-VAD-fmk lead to a 45 % decrease in cell viability within 24 hours with no loss of viability seen in the corresponding wild type MEFs or HEK cells. These results show that autophagy is essential for removal of protein aggregates resulting from the inhibition of peptide N-glycanase.

Further work will focus on the investigation of the autophagic machinery linked to clearance of protein aggregates caused by peptide N-glycanase inhibition.

(126) Specificity of mammalian C-mannosyltransferases for different tryptophan residues of thrombospondin type 1 repeats

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C-mannosylation of tryptophan residues occurs in the endoplasmic reticulum (ER) where it uses the donor substrate Dol-P-Man to modify specific recognition sites occurring in many different proteins. Originally this site was defined as the first tryptophan of the WxxW sequence. In mammalian thrombospondin type 1 repeats, mannosylation can, however, occur on all three sites of the WxxWxxWxxC motif. The *C. elegans* C-mannosyltransferase DPY-19 was only able to modify the first two tryptophans, thus using a WxxW sequence as recognition site. By generating CRISPR/Cas knock outs (single and multiple) in CHO cells and re-expression of mouse enzymes in cells completely lacking C-mannosylation, we now show that one of the mammalian homologs (DPY19L1) has identical activity as the *C. elegans* enzyme, but another homolog (DPY19L3) specifically modifies the third tryptophan of the WxxWxxWxxC motif. This enzyme, present in vertebrates, but not in most invertebrates, obviously evolved to allow triple C-mannosylation of thrombospondin repeats. Whereas we could show that DPY19L1 is required for the mouse netrin receptor UNC5A to reach the cell surface, DPY19L3 didn't seem to be not essential. Thus, mannosylation of two of the three tryptophans was

sufficient to leave the ER and mannosylation of the third site might provide other functions.

(127) Genotype-Phenotype Correlations for POMGNTs in Congenital Muscular Dystrophy

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Congenital muscular dystrophy (CMD) is a class of genetic disorders with phenotypes resulting from hypoglycosylation of α-dystroglycan (α-DG). POMGNT1 and POMGNT2 are glycosyltransferases that modify α-DG. Our work investigates the role of three naturally occurring mutations in POMGNT1, D556N, R311G, and R605H, and in POMGNT2, R157H, G412V, and R445*, to begin to define a genotype:phenotype correlation. All POMGNT2 mutations cause Walker Warburg Syndrome. POMGNT1 mutations R311G and R605H cause Muscle Eye Brain disease, while the D556N mutation in POMGNT1 causes limb-girdle muscular dystrophy, a less severe CMD with no neurological symptoms. To date, the impacts of these mutations on the enzymes' properties are not well established in the literature. To determine the kinetic and stability effects of theses mutations on their respective enzymes, HEK293F cells were transfected with mutant plasmids generated through QuickChange II Site-Directed mutagenesis. While all POMGNT1 mutants and wildtype enzymes expressed in our system, Coomassie-stained gels of purified proteins and relative mRNA levels showed a lack of protein expression for all POMGNT2 mutants. Overnight radiolabel transfer assays were used to establish which POMGNT1 mutants were kinetically dead. Correlating with phenotype, the Limb-Girdle mutant (D556N) showed transfer while the two mutants that cause MEB (R311G and R605H) did not. Next, Promega's UDP-Glo™ assays were used to determine the catalytic effect of the D556N mutation on POMGNT1. D556N showed decreased activity across all concentrations of substrate compared to wildtype POMGNT1. A thermal stability assay was performed on POMGNT1 WT and its mutants to determine whether the mutations affected the stability and folding of the enzymes. Our data indicate that all of the POMGNT1 mutants are thermodynamically stable. We are currently investigating whether the POMGNT1 mutants are capable of binding the sugar nucleotide donor, UDP-GlcNAc, and/or an O-Man peptide acceptor. *In vivo* experiments are being conducted to investigate rescue of POMGNT1 or POMGNT2 null cell lines for IIH6 reactivity, laminin binding, and Lassa pseudovirus entry. A potential application of characterizing genotype-phenotype correlations in these glycosyltransferases is the development of customized treatments for individuals based on the impact of the mutation(s) they carry on enzyme function.

(128) An MPI-independent pathway routes glucose into Mannose-6-P and N-glycans

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Protein N-glycosylation requires the intermediate Mannose-6-P (Man-6-P). It is normally produced either directly from mannose (Man^M-6-P) or, alternatively, from glucose (Man^G-6-P) via phosphomannose isomerase (MPI) (fructose-6-P<–> mannose-6-P), the only enzyme known to catalyze this reversible reaction. Partial MPI deficiency in humans causes Congenital Disorder of Glycosylation MPI-CDG (CDG-Ib), but patients can be treated with mannose (Man) supplements that restore normal glycosylation.

Here we report that even in the complete absence of MPI and Mannose, *Mpi* null MEFs produce sufficient Man^G-6-P to ensure normal protein glycosylation. When labeled with the metabolic tracer [U-^{13}C]Glc, these MEFs incorporate Man^G into newly synthesized N-glycans, suggesting an alternate, MPI-independent pathway for glycan synthesis. Furthermore, labeling *Mpi* null MEFs with [U-^{13}C]Man shows not only the expected intracellular accumulation of Man^M-6-P, but also accumulation of Fru^M6-P and Glc^M-6-P, suggesting an alternate pathway for the reverse MPI reaction, as well. We provide in vitro evidence that human phosphoglucose isomerase (PGI, Fru6P<–>Glc6P) occasionally converts Glc-6-P directly to Man-6-P, bypassing Fru6P, to create an MPI-independent route. This minor reversible reaction occurs at ~0.01% the rate of the normal PGI reaction. Even though this PGI-mediated reaction is rare, the high metabolic flux of Glc needed for energy metabolism provides sufficient Man^G for normal N-glycan synthesis. Consistent with the metabolic flux of Glc-6-P and the amount of Man-6-P needed for N-glycosylation, PGI most likely provides an MPI-independent pathway in MPI null MEFs to supply sufficient Man-6-P for N-glycosylation. In the presence of exogenous Man, Mpi-null MEFs accumulate extremely high concentrations of Man^M-6-P, providing abundant substrate for PGI's reverse reaction and formation of Fru^M6-P and Glc^M-6-P (Supported by The Rocket Fund and R01DK99551).

(129) Extracellular O-GlcNAc is required for retinal vascular development and Dll4-Notch signaling

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Glycosylation on EGF repeats of Notch receptors by O-fucose and O-glucose glycans mediates the strength of Notch signaling required for numerous developmental and physiological processes. In addition, Notch1 receptors are also glycosylated inthe endoplasmic reticulum with O-GlcNAc on EGF repeats by the EGF-domain specific O-GlcNAc transferase (EOGT). Lack of O-GlcNAc caused by homozygous *EOGT* mutation is responsible for a human congenital disease, Adams-Oliver Syndrome. To identify biological and molecular functions of O-GlcNAc, we investigated roles for *Eogt* in retinal angiogenesis. *Eogt*-deficient mice exhibited a hypervascular phenotype and defects in vascular integrity in retinas, which were exaggerated by removing a single allele of *Notch1* or *Rbpj*. Moreover, conditional endothelial cell deletion of *Eogt* also resulted in impaired retinal angiogenesis and vascular integrity. In *EOGT*-deficient cells, the binding of DLL4, but not JAG1 canonical Notch receptor ligands, was reduced and ligand-induced Notch signaling was also impaired. Furthermore, mutagenesis of O-GlcNAc sites on NOTCH1 resulted in decreased binding of DLL4. The combined results provide strong evidence that O-GlcNAc on EGF repeats of Notch receptors mediates DLL ligand-induced Notch signaling and is required for optimal retinal vascular development.

(130) Mapping Modification of O-Glycosylation Sites of Delta-like Proteins

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Dynamic activities of cell-cell signaling transduced by the Notch receptor and its ligands are fundamental to metazoan development. Activation of the Notch receptor is primarily mediated by interactions between the Extracellular Domain (ECD) of Notch and the ECD of its ligands, Jagged1, 2 and Delta-like (DLL) 1, 3 and 4. Previous biological and structural data have implicated glycosylation events on epidermal growth factor-like (EGF) repeats as determinants and modulators of ligand binding and Notch signaling. O-Fucosylation of Thr/Ser residues in Notch EGF repeats by the mammalian Protein O-fucosyltransferase 1 (POFUT1) at the consensus sequence $C^2XXXX(\underline{S/T})C^3$ is necessary for Notch function. Fringe, a b1,3-GlcNAc-transferase, enhances or inhibits Notch activity by elongating O-fucose residues with a GlcNAc that can be further modified by a galactosyltransferase and sialyltransferase in mammals. Overall, signaling assays have demonstrated that Fringe enhances Notch1 activity in the presence of DLL1 and inhibits Notch1 activity in the presence of Jagged1. Rumi, a Protein O-glucosyltransferase, adds a glucose at the consensus sequence $C^1X\underline{S}XP/AC^2$ of EGF repeats, and the O-glucose can be elongated by up to two α1,3-linked xyloses. Although genetic and cell-biological studies suggest that the O-glycan modifications of Notch are important for its activity, Delta-like and Jagged ligands also contain EGF repeats glycosylated by POFUT1, Fringe, and Rumi that may have biological significance. Using a targeted mass spectral approach to analyze glycosylation states of over-expressed Delta-like proteins, we predict to find O-glucosylation and O-fucosylation with possible elongations at the consensus sequences of DLL1, DLL3, and DLL4.

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(131) The Analysis of O-Fucose Glycosylation of Thrombospondin Type 1 Repeats

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Thrombospondin type 1 repeats (TSR's) are small cysteine-rich motifs that are roughly 60 residues in length, are typically found as tandem repeats, and are defined by the presence of 6 highly conserved cysteines which form three disulfide bonds, stabilizing their three dimensional structure. Many of these proteins play critical roles in metazoan development, hemostasis, and the Complement Pathway. Most TSR containing proteins are secreted extracellularly. TSR's from 50 proteins contain the consensus sequence, CXX(S/T)CXXG, which is modified with an unusual but highly important O-Fucose disaccharide: Glcß1-3Fuc. The enzyme that fucosylates TSR's is Protein O-Fucosyltransferase 2 (POFUT2), and the O-fucose can be extended to the disaccharide with b3-glucosyltransferase (B3GLCT). POFUT2 null mice die during early embryogenesis. Based on this embryonic lethality we hypothesize that the O-fucose disaccharide is essential for the function of at least one or more TSR containing proteins. Furthermore, patients with inactivating mutations in B3GLCT suffer from Peters Plus Syndrome (PPS), a Congenital Disorder of Glycosylation resulting in Peters Anomaly (anterior eye chamber defects), cleft lip and/or palate, heart defects, and disproportionate short stature. Both POFUT2 and B3GLCT are localized in the Endoplasmic Reticulum (ER) and recognize properly folded TSR's. Our prior results suggest that O-fucosylation of TSR-containing proteins is essential for their secretion, while modification by B3GLCT affects secretion of some but not all POFUT2 targets. To analyze which POFUT2 targets are sensitive to elimination of POFUT2 or B3GLCT, we have knocked out both enzymes in HEK293T cells and developed secretion assays to test which POFUT2 targets are sensitive to loss of either enzyme. In addition, we hypothesize that patients with Peters Anomaly but without the other phenotypes observed in PPS may not lack B3GLCT but may have defects that interfere with the addition of glucose to O-fucose. Thus, we are

developing a clinical assay to determine if these patients have reduced levels of the disaccharide on Properdin, a protein found in serum containing TSR's which are modified with the O-fucose disaccharide in normal patients, via mass spectrometry. This work was supported by the Georgia Research Alliance.

(132) ST6Gal-I sialyltransferase promotes an anti-apoptotic, cancer stem cell phenotype

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The α2-6 sialyltransferase ST6Gal-I has long been implicated in carcinogenesis, however its pro-tumorigenic function remains unclear. Immunohistochemical analyses from our group reveal ST6Gal-I upregulation in multiple cancers, and high levels correlate with metastasis and reduced patient survival. Contrarily, ST6Gal-I is low in normal epithelia, with the exception of strong expression in stem/progenitor compartments. Furthermore, ST6Gal-I expression is induced upon reprogramming of differentiated cells into pluripotent stem cells. Given these findings, we hypothesize that ST6Gal-I endows tumor cells with a Cancer Stem Cell (CSCs) phenotype. Through manipulating ST6Gal-I expression (overexpression/knockdown) in ovarian and pancreatic cancer cells, we find ST6Gal-I confers hallmark CSC characteristics including tumorspheroid growth, chemoresistance, and upregulation of stem-associated transcription factors. Additionally, ST6Gal-I increases tumor-initiating potential as measured by both limiting dilution assays and a chemically-induced carcinogenesis model implemented with ST6Gal-I-overexpressing transgenic mice. Complementing these findings, recent studies indicate that ST6Gal-I activity enhances the viability of cells exposed to serum growth factor-depleted culture conditions. The ability to survive and proliferate in the absence of growth factors is a key characteristic of tumor cells. High ST6Gal-I expressors display increased activation of survival indicators (pAkt, cIAP2, pNFkB), but reduced cell death markers, under serum-depleted growth conditions. Moreover, cells with high ST6Gal-I expression display enriched levels of cyclin D2 and a greater percentage of cells in S phase. These data suggest that ST6Gal-I activity is critical for maintaining tumor cells in a proliferative state following exposure to serum deprivation. When coupled with our prior work showing ST6Gal-I-dependent protection against death receptor (Fas, TNFR1) and galectin-dependent apoptosis, these results establish ST6Gal-I as a fundamental survival factor. In tandem with ST6Gal-I overexpression/knockdown models, we observe consistent clonal selection for cells with high endogenous ST6Gal-I when cells are exposed to stressors including tumorspheroid culture, multiple chemotherapeutics, growth factor deprivation, and death receptor ligands (TNFα). Collectively these results point to a pervasive role for ST6Gal-I in driving tumor cell resistance to numerous death-inducing stimuli within the tumor microenvironment.

(133) Cell Surface Glycoprotein Aging and Turnover Modulates a Constitutive Anti-Inflammatory Mechanism of Host Protection that is Progressively Disabled by a Foodborne Pathogen

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Intestinal inflammation is the central pathological feature of colitis and the inflammatory bowel diseases. These syndromes arise primarily from unidentified environmental factors. We have discovered that sub–lethal oral infection of mice with *Salmonella enterica* Typhimurium (*ST*), a major pathogen source of human food poisoning, caused a progressive inflammation of the intestinal tract that persisted following pathogen clearance and escalated with recurrent infections. In this model of human food poisoning, *ST* infection disabled a previously unknown protective mechanism in the host that maintains intestinal alkaline phosphatase (IAP) function. The lipopolysaccharide endotoxin present in Gram-negative bacterium such as *ST* recapitulated the induction of host Neu3 neuraminidase by *ST*, and thereby accelerated the molecular aging and turnover of nascent IAP at the enterocyte cell surface. Uninfected mice genetically deficient in the ST3Gal-VI sialyltransferase and resulting IAP sialylation exhibited similar inflammatory disease signs. Oral administration of IAP or the neuraminidase inhibitor Zanamivir was therapeutic with reduction and elimination of disease signs of intestinal inflammation and tissue damage. These discoveries reveal that an environmental pathogen disrupts a constitutive host anti-inflammatory mechanism linked to the regulation of cell surface IAP glycoprotein aging and turnover.

(134) Galectin-8 as a regulator of bone remodeling and osteoporosis

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Bone remodeling, the balance between bone formation and resorption, is maintained by the coordinated activity of osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells. We have recently shown that mice overexpressing galectin-8, a secreted animal lectin of the galectins family, exhibit accelerated osteoclasts activity and bone turnover, which culminates in reduced bone mass, similar to cases of postmenopausal osteoporosis. Based on

these findings, we wished to determine whether there are common mechanistic elements between galectin-8 over-expression and postmenopausal estrogen deficiency, both leading to osteoporosis. To this end, we performed ovariectomy to induce estrogen deficiency in mice. Mice were analyzed 6 and12 weeks post operation. The ovariectomized wild-type mice (WT-Ovx) and the sham-operated galectin-8 over-expressing mice (Tg-gal8 mice), indeed showed a similar pattern of early onset osteoporosis, as measured by the kinetics and fold changes in bone loss, e.g 46% and 37% reduction in bone volume to tissue volume ratio (BVF) of WT-Ovx and Tg-gal8 mice, respectively. Ovariectomy of Tg-gal8 mice led to even greater bone loss (63% reduction in BVF). This phenotype could be attributed to two opposing effects of galectin-8 and estrogen on bone remodeling. First, galectin-8 promotes osteoclast differentiation by inducing the secretion of RANKL from osteoblasts, a process that is known to be inhibited by estrogen. Second, we found that galectin-8 inhibited the differentiation of osteoblast into mature, bone forming cells, in a cell-autonomous manner, by inhibiting BMP signaling, a major signaling pathway governing osteoblast differentiation. Such inhibition was manifested by a reduction in Runx2, the master regulatory factor of osteoblast differentiation, as well as a reduction in BMP signaling targets and the ability of such osteoblasts to create mineralized bone. In contrast, estrogen is a known promoter of osteoblasts differentiation. Taken together, our findings identify galectin-8 as a new player in osteoclastogenesis that can counter-act the activity of estrogen. Therefore, the combined effects of ovariectomy and overexpression of galectin-8 synergize in promoting bone loss. Our findings therefore identify galectin-8 as a new promoter of osteoporosis.

(135) The Effect of Polysaccharides from Karenia mikimotoi on CAM Angiogenesis

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Karenia mikimotoi is a kind of bloom micrioalgae which can often result in red tide over the world. Some reports focused on its toxin and the control. The effect of polysaccharides from *Karenia mikimotoi* (KPS) on chicken chorioallantoic membrane (CAM) angiogenesis was studied. KPS was obtained by flash extraction combinedwith alkali protease enzymolysis and theoptimalprocessconditions were determined as 40°C, pH8.5, 2.5%and 2hfor enzymolysistemperature, pHvalue, enzymolysisdosage andextraction time with single factors and orthogonal experiments. The karenia polysaccharides were obtained by using trichloroacetic acid for getting rid of protein and 30%, 50%% and 80% ethanol for precipitation to get different polysaccharides (named as KPS1, KPS2 and KPS3, respectively). The effect of KPS1 on vessel growth rate was only 44.3%, 66.7% at the concentration of 100 and 200 $\mu g \times mL^{-1}$, respectively. While the effect of KPS2 could reach 56.8% and 57.9% at the concentrations of 100 and 200 $\mu g \times mL^{-1}$ when setting the vessel growth rate was 100% by adding PBS solution as blank control. KPS3 had no significant effect on vessel growth rate compared with the control. The results indicated that KPS1 and KPS2 had anti-angiogenic potentiality. The experiments were supported by National Natural Science Foundation of China (No. 41306137) and Supported by Program for Liaoning Excellent Talents in University (No. LJQ 2014078).

(136) The Anti-angiogenic Activity of Polysaccharides from Chlorella spp

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Chlorella vulgaris is a common kind of micrioalgae with abundant biological bioactivitors such as unsaturated fatty acid, protein, polysaccharides and pigment, which have anti-virus, anti-tumor, anti-bacteria, radiation-resistance and so on. The anti-angiogenic activity of chlorella polysaccharide (CPS) was studied by using CAM and the proliferation and migration of induced human umbilical vein endothelial cells models. The polysaccharides were prepared with flash extraction combinedwith enzymatic method. The optimal process conditions were determined as 1.5% trypsase. pH7.5, 40°C and 2h for enzyme dosage, pH value, temperature and extraction time. CPS were obtained by using trichloroacetic acid for getting rid of protein and 30%, 50% and 80% ethanol for precipitation to get different polysaccharides (CPS1, CPS2 and CPS3). CPS2 had significant inhibition on vessel growth at the concentration of 100 and 200 μg×mL-1, respectively, and the vessel growth rate was only 62.9% and 50.5% when setting the rate was 100% by adding PBS solution as blank control. CPS3 had some inhibition at the both concentrations. CPS1 and CPS2 had certain inhibition on the proliferation and migration of induced human umbilical vein endothelial cells at the concentration of 5-200μg×mL-1. The result indicated that chlorella polysaccharide had anti-angiogenic activity which showed the inhibition of tumor migration. The experiments were supported by National Natural Science Foundation of China (No. 41306137) and Supported by Program for Liaoning Excellent Talents in University (No. LJQ2014078).

(137) Antibacterial membrane attack by a pore-forming of manila clam Ruditapes philippinarum lectin

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Earlier, the antibacterial activities of manila clam *Ruditapes philippinarum* lectin (MCL) were observed and studied. Unfortunately there are limited data about MCL recognize

their bacterial targets by binding peptidoglycan carbohydrate. Here we study the mechanistic basis for MCL antibacterial activity. The results showed that MCL inhibited the growth of *Shewanella* sp., which was dependent on temperature. *Shewanella* sp. treated with MCL showed that the cell membrane was wrinkled and formed more irregular pores at 30°C than at 4 °C. In the presence of MCL, the changes of Ca^{2+} of *Shewanella* sp. were analyzed. *Shewanella* sp. treated with MCL showed lower intracellular calcium concentration than the control group. This could be because MCL disturbed the ordered arrangement of membrane lipid and changed permeability of plasma membrane, with irregular pores appearing in the plasmalemma and Ca^{2+} of the cells leaking out. This work was supported by a grant from the National Natural Science Foundation of China (31571916).

(138) The anti-hyperglycemic activity of a polysaccharide from Crassostrea gigas in alloxan induced diabetes in ICR mice

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Oyster has been used in traditional Chinese medicine for diabetes treatment. But the anti-hyperglycemic effective sites of a parts from *Crassostrea gigas* were not exposed. A water-soluble polysaccharide with a uniform glucose polymer was isolated from *C. gigas* (CGPS-1) and the anti-hyperglycemic activity of CGPS-1 was investigated in both normal health and alloxan induced diabetes in ICR mice. CGPS-1 was orally administered once a day after 3 days of alloxan-induction at 200, 400 and 800 mg/kg body weight for 28 days. Treatment with CGPS-1 decreased fasting glucose level, and the glucose tolerance was marked improvement in loss in ICR mice. CGPS-1 of 400 mg/kg displayed the optimal anti-hyperglycemic effect on diabetic mice and reduced GSP, TCH and TG levels of diabetic mice. The protective effect of CGPS-1 on loss in body weight was investigated in ICR mice. In addition, CGPS-1 did not exhibit any toxic symptoms in the limited toxicity evaluation in mice. This work was supported by a grant from the Marine Public Welfare Research Project (201405017-03).

(139) Mass spectrometry analysis of adeno-associated virus glycan receptor expression in aging striatum for gene therapy

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Parkinson's disease (PD) is a neurological disorder characterized by the degeneration of dopaminergic neurons in the nigrostriatal pathway in the brain. Gene therapy is an avenue for treatment with potential to improve the dopamine production or provide neuroprotective factors to prevent further degeneration of neurons in PD patients. Currently, adeno-associated viral vectors are being used in clinical trials to deliver trophic factors to the striatum of PD patients in order to provide neuroprotection for the remaining dopamine neurons. Adeno-associated viruses (AAV) are vectors of choice in gene therapy due to their well characterized safety and efficacy profiles. Significantly, all primary receptors of the virus are glycans. While gene therapy has been successful in preclinical PD models using young adult rats and monkeys, clinical trials in aged PD subjects have failed. We hypothesize that age-related changes in glycan receptors in neurons and/or extracellular matrix (ECM) results in decreased adeno-associated virus binding. Further, the differential expression levels and varied sulfation patterns of viral capsid receptors of the ECM components of heparan sulfate (HS) proteoglycans (receptor for recombinant AAV2) differ with age in the rat striatum, potentially affecting transduction efficiency. Method: We analyzed glycan classes and proteins from rat brain histological sections using liquid chromatography-mass spectrometry. Results: We observed higher expression of N-sulfated HS disaccharides in aged (20 months) compared to young (3 months) rat striatum. In particular, the receptor for the virus is NS, 6S sulfated patterns with corresponding disaccharide compositions of D0S6 and D2S6 forms. We observed that the abundance of this receptor is higher in old compared to young striatum with reduction in transduction levels of rAAV2/2 in aged brains. This implicates possible sequestration of the virus in ECM of aged brains with corresponding reduction in transduction. Further studies in human brain will enable better understanding to allow for improved design of viral vectors for success of gene therapy in clinical trials in aged PD subjects.

(140) High-throughput sequential glycoprofiling of six abundant glycoproteins IgG, IgA, IgM, transferrin, haptoglobin and alpha-1-antitrypsin in ovarian cancer

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Ovarian cancer is the most lethal of all gynecological cancers among women. Serum CA125 is the only biomarker that is used routinely and there is a need for further complementary biomarkers both in terms of sensitivity and specificity. Cancer is associated with altered expression of glycoproteins and detecting changes in their *N*-glycans can be useful for insight into disease pathogenesis and for cancer

biomarkers discovery. Changes in glycosylation of immunoglobulins IgG, IgM and IgA and the acute phase proteins haptoglobin, alpha-1-antitrypsin and transferrin have been reported in ovarian cancer and may contribute to disease pathogenesis.[1, 2]

We will present the first automated platform for sequential *N*-glycan analysis of six glycoproteins using Ultra High-Performance Liquid Chromatography for up to 96 samples in tandem: IgG, IgM, IgA, transferrin, haptoglobin, alpha-1-antitrypsin in an ovarian cancer set and healthy controls (n=37). We will utilize a novel tip technology with custom packed antibodies for sequential affinity chromatography of the glycoproteins from the biological matrix (serum) followed by *N*-glycan purification using ultrafiltration and fluorescent labeling with 6-aminoquinoline carbamate, previously described by our group.[3] We will provide insight into contributions from the individual immunoglobulin and the acute phase protein *N*-glycans to the disease and use statistical tools to present potential biomarkers for ovarian cancer cohort. Statistical models will also be presented to show differentiation between different classes of ovarian cancer including normal, borderline, malignant, metastatic, and unclassified.

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(141) Depletion of sialic acid in podocytes results in kidney failure

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The role of sialic acid (Sia) in kidney development and function is still not fully understood. The activation of sialic acid to CMP-Sia prior to the synthesis of sialoglycans is catalyzed by the nuclear enzyme CMP-Sia synthetase (CMAS). An overall reduction of the CMAS expression level in *Cmas*^{nls} mice resulted in kidney failure within three days after birth [1]. A heavy proteinuria pointed towards a defect in the glomerular filtration barrier which is composed of three layers: the fenestrated endothelium, the glomerular basement membrane and the slit diaphragm, a protein complex between adjacent podocyte foot processes. The lethality in *Cmas*^{nls} mice could be attributed to pathological changes in podocytes forming the outermost layer of the filtration barrier. For further analysis we generated a podocyte-specific *Cmas* knockout mouse model (*P-Cmas*$^{-/-}$). These mice mimic nephropathies such as *Focal Segmental Glomerulosclerosis* (FSGS) and die within two month after birth. They develop massive proteinuria and show over 20-fold increased urinary protein/creatinine levels 42 days after birth. Similar to *Cmas*^{nls} mice, the development of the disease is accompanied by a progressive loss of Sia on nephrin and podocalyxin, two major sialoglycoproteins of the podocyte. Electron microscopy revealed that in knockout animals the podocyte morphology was drastically disorganized with effaced podocyte foot processes and a loss of slit diaphragms. To investigate the role of Sia for podocyte morphology and function on the cellular level, we first generated an immortalized wildtype podocyte cell line. These cells are able to proliferate in culture and can be differentiated into an *in vivo*-like state. Subsequently, we generated a *Cmas* knockout cell line using the CRISPR/Cas system. The knockout podocytes are viable and show wildtype morphology, but appear to proliferate slower than the wildtype. The biochemical characterization of these cells and the impact of sialylation for podocyte proteins like podocalyxin and nephrin are currently under investigation.

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(142) Nutrient Regulation of Signaling & Transcription by O-GlcNAc

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O-GlcNAc cycles on and off thousands of nucleocytoplasmic proteins, has extensive crosstalk with protein phosphorylation and is fundamentally involved in nearly all aspects of transcription & signaling. O-GlcNAc regulates the cycling of the TATA-binding (TBP) protein on and off DNA during the transcription cycle and is required for TBP to bend DNA. Targeted, inducible, deletion of the O-GlcNAc Transferase in CAMKII positive (excitatory) neurons of adult mice results in a morbidly obese mouse with a satiety defect. Thus, O-GlcNAcylation is directly involved in appetite regulation. More than one-half of all human protein kinases are modified by O-GlcNAc and all kinases that have been tested are indeed regulated by the sugar. Abnormal O-GlcNAcylation of CAMKII contributes directly to diabetic cardiomyopathy and to arrhythmias associated with diabetes. Over eighty-eight O-GlcNAcylated proteins have been identified in cardiac mitochondria and many are in the electron transport chain. In diabetic rats, not only is the O-GlcNAcylation of mitochondrial proteins increased, but also the O-GlcNAc transferase is mislocalized leading to grossly altered patterns of O-GlcNAcylation and directly causing mitochondrial dysfunction. This is the first direct molecular link explaining why hyperglycemia causes mitochondrial dysfunction. Also, in diabetic rats, hyper-O-GlcNAcylation of transcription factors blocks the expression of podocin

and nephrin, two key components of the filtration apparatus (Slit diaphragm) of the kidney, contributing directly to diabetic nephropathy. Prolonged elevation of O-GlcNAc, as occurs in diabetes, contributes directly to diabetic complications and is a major mechanism of glucose toxicity. Supported by NIH P01HL107153, R01DK61671 and N01-HV-00240. *Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU.*

(143) The identification of allosteric mechanisms allows utilizing conserved enzymes as novel drug targets

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UDP-glucose (UDP-Glc) is an essential metabolite in all kingdoms of life and the precursor for other - likewise essential - activated sugars which are utilized in a plethora of biological functions. UDP-Glc synthesis is catalyzed by UDP-glucose pyrophosphorylase (UGP), an enzyme that is highly conserved among eukaryotes. Unicellular pathogens such as *Leishmania* and *Trypanosoma* - causative agents of Leishmaniasis, Sleeping Sickness and Chagas disease – collectively cause up to 100.000 deaths annually. These pathogens depend on UDP-Glc and its derivatives for virulence and viability, making enzymes involved in UDP-Glc synthesis potential drug targets. This strategy is, however, challenged by the high conservation that exists on primary, secondary and tertiary structure level between UGPs from eukaryotic parasites and their human orthologs. Therefore, selective inhibition of the pathogen enzymes is unlikely to be achieved by targeting the active site. To establish a rational basis for the development of alternative ways of inhibiting the pathogen UGP, we comparatively characterized UGP from *Leishmania major* and human at both structural and functional level. We found that monomeric *L. major* UGP facilitates an intramolecular mechanism of substrate coordination which is crucial for activity and requires major conformational flexibility. In contrast, substrate coordination in the octameric human UGP, in which subunits are overall more rigid, is achieved in an intermolecular, quaternary fashion. Consequently, we identified unique, dynamic allosteric sites in the *L. major* enzyme which are linked to enzymatic activity. Their inhibitory potential was confirmed by site-directed mutagenesis. Guided by structural and experimental insights, an inhibitory lead compound was developed which exhibits an IC_{50} in the micromolar range under *in vitro* conditions. Current work focuses at rationally improving the compound towards increased efficacy and selectivity. Our study serves as a blueprint for future approaches towards structure- and mechanism-based drug design, allowing to target enzymes that are conserved between pathogens and their hosts. Interestingly, also bacterial UGPs are proposed to be attractive drug targets and could provide a promising new approach to circumventing the increasing problem of antimicrobial resistances. The fact that eukaryotic and prokaryotic UGPs show no significant sequence similarity increases the likelihood of identifying unique inhibitory sites.

(144) Glycomic analysis of gastric carcinoma cells discloses glycans as modulators of RON receptor tyrosine kinase activation in cancer

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The upregulation of terminal sialylated glycan epitopes such as $SLe^x$is a common event in gastric carcinogenesis and cancer progression. Our understanding on the molecular mechanisms underlying aberrant glycosylation and the phenotypic consequences is limited. In an attempt to shed light on these mechanisms we have established a gastric carcinoma cell line stably transfected with the glycosyltransferase ST3GAL4 which led to the upregulation of sialylated epitopes such as SLe^x. The cell line model shows increased invasive capacity both in vitro and in vivo. This aggressive behavior resembles the phenotype observed in patients with gastric cancer overexpressing SLe^x.

We evaluated at the structural level the glycome and the sialoproteome of this gastric cancer cell line utilizing state-of-the-art analytical methods such as HILIC-FLD-UPLC and LC-ESI-MS/MS (1, 2). Our results showed that the overexpression of ST3GAL4 leads to a broad range of glycomic changes on both *N*- and *O*-glycans. The alterations included reduced extension of O-glycans, reduction of bisecting and increase of branched structures on *N*-glycans (3), and a shift from α2,6- towards α2,3-linked sialylated *N*-glycans, which was also confirmed by lectin blotting. Target proteins carrying aberrant glycosylation were identified by quantitative sialoproteomic

analysis of isobaric labeled glycopeptides. We identified 47 proteins with significantly increased sialylated *N*-glycans. These included Integrins, Insulin receptor, Carcinoembryonic antigens and RON receptor tyrosine kinase, which are proteins known to be key players in malignancy.

Further analysis of RON confirmed its modification with SLe^x and the concomitant hyperactivation of this receptor. We finally revealed overexpression and co-localization of SLe^x and RON in human gastric tumors. Our data support that cellular aberrant glycosylation is an alternative mechanism of the oncogenic RON receptor tyrosine kinase activation, with potential clinical applications.

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(145) Chondroitin sulfate analysis of myelinated versus non-myelinated regions of human brain tissue

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Chondroitin sulfate proteoglycans (CSPGs) and hyaluronan aggregates compose a significant portion of the brain extracellular matrix. CSPGs are involved in crucial brain processes such as brain connectivity, axon guidance, impulse conduction and myelination. Furthermore, CSPG abnormalities have been reported in subjects with schizophrenia (SZ), in which thalamo-cortical disconnectivity has been repeatedly reported in brain imaging studies. We proposed that CSPG expression in the thalamus thus may play an essential role in the disruption of thalamo-cortical connectivity. Preliminary immunohistochemistry results on the mediodorsal nucleus (MD), a large thalamic nucleus, of SZ subjects showed altered organization of myelinated fiber bundles. Also, glycomics studies on SZ and control MD tissues showed a significant increase in unsulfated CS disaccharide in SZ, compared to healthy controls. In this study, our aim was to test the hypothesis that CSPGs, myelin, and related extracellular matrix components are altered in subjects with SZ. To date, no studies have compared the CS abundance in myelinated *versus* non-myelinated regions

To test this, preliminary glycomics analysis was performed on myelinated fiber bundles and non-myelinated regions of the thalamus sections from healthy controls. The CS disaccharides were released using chondroitinase ABC enzyme by manual on-slide digestion on myelinated and non-myelinated spots of brain sections and analyzed using liquid chromatography-mass spectrometry analysis. Preliminary results showed an increase in total and unsulfated CS disaccharide abundance in myelinated fiber bundles when compared to non-myelinated regions suggesting differential CS patterns in myelin bundles. We plan follow-up studies using samples from SZ patients and control subjects in order to compare myelinated and non-myelinated regions of the MD thalamus. Information obtained from extensive medical records available for these subjects will be used to test for potential effects of confounding factors, including antipsychotic treatment, lithium, antidepressants, age, sex, cause of death, etc. This information will contribute to the understanding of the molecular and neuropathological underpinnings of thalamo-cortical disconnectivity in SZ.

(146) Role of Fucosyltransferase 8 in Pathogenesis of Epidermal Proliferation/Differentiation and Psoriasis Development

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Psoriasis is characterized by excessive growth and aberrant differentiation of keratinocytes. In recent years, different genes and proteins have been highlighted as potential biomarkers for psoriasis, but most of these are not psoriasis specific. The pathogenesis of psoriasis remains poorly understood, and the effectiveness of disease treatments still leaves much to be desired.

We investigated the role of mammalian α1, 6-fucosyltransferase (FUT8) in psoriasis epidermal development in human samples, animal models, and human keratinocyte cell-line. FUT8 catalyzes the transfer of a fucose residue from a donor substrate, GDP-fucose, to the N-linked acetylglucosamine of the core structure complex glycopeptides. Core-fucosylation, or α1,6-fucosylation, plays an essential role in various pathophysiological events, such as cell growth. We found that in both human and animal skin samples, FUT8 and its core-fucosylated glycoproteins were correlated with skin epidermis proliferation and the formation of psoriasis phenotypes. Using skin epidermis from human samples and mass spectrometry methodologies, we identified various α1,6-core-fucose glycoproteins, which were involved in the regulation of keratinocyte proliferation and differentiation. We constructed FUT8 knockdown/overexpressed clones to investigate the

mediating function of FUT8 in the dimerization of epidermal growth factor receptor (EGFR), a cell surface receptor-linked tyrosin kinase for extracellular ligands. The binding affinity between ligands and EGFR was also found to be affected by FUT8. We further illustrated the downstream EGFR signaling pathways, such as PI3K/Akt, MAPK/ERK and NF-κB, which were significantly regulated by FUT8 in human keratinocyte. PI3K/Akt and MAPK/ERK are involved in cell proliferation and differentiation, whereas NF-κB is a key modulator of immunity and inflammation. These findings revealed the complex regulatory mechanisms and dynamic functions of EGFR in keratinocyte, which was heavily modulated by core-fucosylation.

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

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

(148) Accelerated Aging and Turnover of Host Anti-Inflammatory Enzymes Contributes to the Pathogenesis of Gram-negative Sepsis

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Recent discoveries by this laboratory identified a mechanism of secreted protein aging and turnover that is composed of glycosidase–mediated N-glycan remodeling (Yang *et al.*, 2015). We now find that this mechanism is modulated in mouse models of Gram-negative sepsis caused by infection with the human bacterial pathogens *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. During the onset of sepsis, we have measured an increased rate of N-glycan remodeling with ensuing endocytic lectin ligand formation among secreted proteins in the blood plasma, resulting in rapid decreases in the abundance and function of key host anti-inflammatory enzymes, namely tissue non-specific and intestinal alkaline phosphatases (TNAP and IAP). Our findings demonstrate that both of these secreted anti-inflammatory protein enzymes are thereby post-translationally regulated as a means of determining their concentrations and activities in the blood. Our data further reveal that the endogenous sialyltransferase ST3Gal-VI is responsible for TNAP and IAP sialylation, without which both alkaline phosphatase isozymes are rapidly cleared from circulation by the Ashwell-Morell receptor (AMR). This accelerated remodeling of TNAP and IAP in sepsis is due to the induction of neuraminidase activity in the blood. This induction includes Neu1 and Neu3 and can be recapitulated by the lipopolysaccharide component of Gram-negative bacteria, thereby accelerating the aging and turnover of the anti-inflammatory enzymes TNAP and IAP. We further show that the induction of Neu activity with the subsequent reduction of alkaline phosphatase activity are responsible for increased LPS-phosphate levels linked with inflammation and reduced survival of Gram-negative sepsis. These findings demonstrate unexpected features of pathogen and host interactions during sepsis that target an intrinsic host mechanism of secreted protein aging and turnover. The resulting rapid changes in protein abundance and function among the secreted proteome have significant impacts on the pathogenesis and survival of sepsis.

(149) Protein-specific polysialylation: Bringing a biophysical dimension to the biochemical evidence

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Polysialic acid (polySia) is a unique post-translational modification found on a small set of cell-surface glycoproteins, synthesized by Golgi-localized polysialyltransferases (polySTs). This large, negatively charged glycopolymer is capable of

abrogating cell-cell adhesion, it modulates signaling of the substrates it modifies and functions as a reservoir for growth factors and neurotransmitters. PolySia is crucial for the proper development of the nervous system, tissue regeneration, and is also upregulated in numerous late-stage, highly aggressive cancers. Studies in our laboratory concerning the sequence requirements for protein-specific polysialylation show that the polySTs recognize an acidic region on a "recognition domain" of each substrate that then allows these enzymes to polymerize polySia on glycans of an adjacent domain or region. Previous studies on the Neural Cell Adhesion Molecule, NCAM, have demonstrated that its first fibronectin III repeat (FN1) is its recognition domain and polyST binding to this region allows the polysialyation of N-glycans on the adjacent immunoglobulin domain (Ig5). In the present work, we show that this paradigm holds for another substrate Neuropilin-2 (NRP-2). We find that acidic residues in the MAM domain of NRP-2 are required for the polysialylation of its O-glycans in the adjacent linker region. We have also identified a polybasic region (PBR) in the polySTs that contains basic residues required for NCAM polysialylation. We will present competition studies that demonstrate that overlapping sets of basic residues in the PBR of ST8SiaIV and ST8SiaII are required for recognition of their substrates, NCAM, NRP-2 and SynCAM-1. In addition, we will provide direct biophysical evidence from ITC and NMR studies that demonstrate that two basic residues of the ST8SiaIV PBR are required for binding to FN1 and confirm the involvement of an extended acidic patch on FN1 as well as other residues in this interaction. These observations establish the mechanism of protein-specific polysialylation and provide the basis for polyST-substrate interaction inhibitor design. This work was supported by NIH RO1 GM101949 (to KJC).

(150) Defining the OGT interactome: a lesson in survival

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The addition of O-linked N-acetyl-β-D-glucosamine (O-GlcNAc) to serine and threonine residues is a dynamic post-translational modification involved in a variety of cellular processes. Previous research has demonstrated that cellular stress elevates global O-GlcNAc levels, and this response is thought to mitigate injury and promote cytoprotection. The O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA) are the sole enzymes that add and remove O-GlcNAc from thousands of substrates. Although several isoforms exist for each enzyme, it remains unclear how the cycling of O-GlcNAc can be controlled in a precise fashion to affect the correct targets and signaling pathways both basally and in response to stress. One mechanism by which OGT may be regulated is through protein interactions that ultimately affect activity, localization and substrate specificity. To provide insight into the regulation of OGT, we have utilized quantitative proteomics to define OGT's basal and stress-induced interactome. Mouse embryonic fibroblasts were labeled with stable isotopes of amino acids in cell culture (SILAC) followed by treatment with hydrogen peroxide. OGT and its interaction partners were immunoprecipitated from OGT wild-type (WT), null and hydrogen peroxide treated cell lysates. Following trypsin digestion, peptides were run on an Orbitrap Fusion Lumos mass spectrometer. Spectra were searched against a murine RefSeq database using Sequest and Mascot and analyzed with Proteome Discoverer. 194 proteins were identified above background in wild-type cells, and an additional 34 proteins were identified in the stressed population alone. Quantitation suggests that at least 60 proteins have a stress-induced association with OGT. A subset of interactors have previously been characterized, such as HCF1 and GAPDH, whereas other proteins, like Mic60, are novel. These data likely highlight specific pathways involved in the oxidative stress response regulated by OGT, as well as protein interactors that mediate OGT activity to direct glycosylation and thus survival. These findings, combined with additional functional studies, should provide a framework for understanding the regulation of OGT in pathophysiological models related to stress.

(151) Glycosphingolipids involved in contact inhibition of cell growth

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Contact inhibition of cell growth, the inhibition of cell proliferation by tight cell-cell contact, is a fundamental characteristic of normal cells that is lost in malignant cells. The cell-density dependent inhibition of growth is considered to be mediated through the cell surface membrane. Our previous study with human embryonic lung fibroblast WI38 cells demonstrated that ganglioside GM3 is involved in contact inhibition. Our preliminary experiments showed certain glycosphingolipids (GSLs), namely GD3 and Gb4 are also involved. In the present study we further investigated the involvement of GSLs in contact inhibition using human epithelial mammary gland MCF10A cells, which show contact inhibition of growth. The expression levels of ganglio- and globo- series GSLs were compared in MCF10A cells at sparse and confluent growth stages by flow cytometry and TLC immunostaining. We found that expression levels of GD3 and Gb3 were significantly increased in the confluent monolayer cells. Quantitative RT-PCR analysis for mRNA levels of ganglio/globo series glycosyltransferases showed

that ST8SIA1(GD3 synthase) and A4GalT (Gb3 synthase) are upregulated in the confluent monolayer cells. We investigated a possible functional role of the GSLs in contact inhibition by exogenous addition of the GSLs and knock-down of mRNA levels of ST8SIA1 and A4GalT by siRNA. Sparsely growing cells treated with the GSLs showed inhibition of cell proliferation detected by BrdU incorporation and MAPK activation. The knock-down of mRNA levels of ST8SIA1 and A4GalT reduced the expression of GD3 and Gb3, respectively; and cell proliferation in the confluent cells was increased as detected by BrdU incorporation and MAPK activation. The Hippo pathway, one of the signaling pathways implicated in the contact inhibition of growth, consists of a kinase cascade with associated regulatory and scaffolding proteins. It has been shown that some membrane-associated proteins, like Merlin/NF2 tumor suppressor protein, function as upstream activators of the Hippo pathway. We will discuss a possible interaction between the GSLs with Merlin/NF2 in membrane microdomains which may result in cell growth inhibition.

(152) Biochemical characterization of Cosmc, a client specific endoplasmic reticulum chaperone

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The O-GalNAc type modification on glycoproteins plays a critical role in many cellular processes, including signaling and communication, cell adhesion, immune surveillance and host-pathogen interactions, inflammation, and endocytosis. Cosmc is an essential endoplasmic reticulum chaperone protein necessary for normal O-GalNAc glycosylation by regulating the activity of its client protein, T-synthase. The glycosyltransferase T-synthase synthesizes the core 1 structure from the precursor Tn antigen (GalNAca1-Ser/Thr), by addition of a galactose to GalNAc-containing polypeptides. Loss of Cosmc or T-synthase results in abnormally truncated O-GalNAc glycans that terminate in the Tn antigen, which is associated with multiple human diseases including cancer. Despite its essential nature, little is known about Cosmc structure, domain organization, mechanism of function, and functional hotspots. Here we characterize Cosmc and two mutant proteins: E152K, a loss of function point mutant identified in patients with Tn syndrome, and CosmcΔ256, an engineered truncation mutant. Cosmc is comprised of two domains, as revealed by limited proteolysis experiments. Deletion of 62-residues from the C-terminus results in CosmcΔ256, which is fully folded by circular dichroism spectroscopy and shows increased thermal stability relative to the wild type. Additionally, both CosmcΔ256 and Cosmc E152K are functional in an *in vitro* assay of chaperone activity. The oligomerization of Cosmc was characterized by chemical crosslinking and blue native page. Cosmc and CosmcE152K can form dimers and higher order oligomers, but not CosmcΔ256. Additionally, Cosmc binds selective divalent cations in thermal shift assays. Metal binding is abrogated by the CosmcΔ256 truncation, but unperturbed by the E152K mutation. Our results suggest that the N-terminal domain of Cosmc mediates T-synthase binding and chaperone function, whereas the C-terminal domain is important for oligomerization and metal binding. The biological roles of oligomerization and metal binding are unclear, but may be important *in vivo* in regulating protein function.

(153) A new anti bis-Tn antibody illustrating the usefulness of a new technological platform using a combination of phage display technique and glycopeptide array.

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Therapies based on antibodies have often turned out successful and there is a rising number of approved antibody based drugs. A clear majority of these antibodies are recognising peptide epitopes on upregulated proteins and avoiding epitopes including post-translational modifications (PTM) such as O-glycosylation. Low affinity and specificity are problems that are associated with the existing antibodies and the overall immune response against this type of hydrophilic and flexible PTM-epitopes. Addressing these issues together with improving the cost effectiveness of development/production is crucial in the generation of novel antibodies.

By using a combinatory antibody-antigen microarray for direct screening of multiple single chain antibody (scFv) clones with no need for pre-purification or enrichment steps before screening, both time and costs are reduced. Two different analyses are performed on the same slide in a spot-on-spot print where the antigen is presented in solution as well as in immobilized form. The ability of the individual scFv clone to bind the soluble form of the antigen favours selection for higher affinity rather than avidity while the binding of scFv to a densely presented immobilized antigen, also enables identification of clones with lower affinity. This approach thus results in an overall increased hit rate. Using this combined approach, we recently identified a Tn-antigen binding scFv of high quality. It was characterised by using both synthetic glycopeptide libraries and biological material. Evaluations showed a clear preference towards double Tn-epitope with binding of GalNAc-Serine and GalNAc-Threonine in all combinations and showing no or very weak binding to single Tn-epitopes. Carbohydrate microarray

analyses showed no binding to sialyl-Tn, blood group A or any of the other tested carbohydrates. Which verify it to be a bis-Tn-specific scFv clone with little intervention from the adjacent peptide backbone. Analysis of the scFv clone binding to Tn-positive Jurkat cells by flow cytometry and western blot showed strong binding and internalization of the scFv, demonstrating its functionality on biological material as well as synthetic material.

(154) Development of Defined Human Chimeric anti-Tn Monoclonal Antibody

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In tumors, emergence of tumor-associated carbohydrate antigens (TACAs) can contribute to tumor progression and metastasis, and potentially be useful as biomarkers or for targeted therapy. One major TACA is the Tn antigen, a truncated mucin type O-glycan (GalNAc-alpha1-O-R, where R is Ser or Thr in a glycoprotein) that is expressed in the majority of carcinomas. Many of the studies examining Tn expression notably rely on various lectins, including VVA and HPA, which bind to GalNAc and other determinants and are not specific to the Tn antigen.

Here we generated a defined human chimeric anti-Tn antibody based on a murine anti-Tn monoclonal antibody (Ca3638, BaGs6) that was previously generated [Springer GF *et al*, *Cancer* 1985 andAvichezer D *et al*, *Int J Cancer* 1997]. We purified Ca3638 (IgM) from mouse ascites fluid and determined the whole antibody sequences by *de novo* proteomic sequencing. We then reverse engineered and humanized the antibody to generate Remab6 (hIgG1). Tn glycopeptide array studies demonstrated that Remab6 recognizes di- or tri-Tn clustered structures on mucin tandem repeats, but binds weakly to Tn on human IgA1-derived glycopeptides, blood group A, or glycans expressing terminal GalNAc other than the Tn antigen. Remab6 bound to human colon and breast cancer cell lines that express the Tn antigen, and exhibited cell killing activity *in vitro*. We are further examining the specificity of Remab6 by immunohistochemistry using a variety of mouse tissues and human cancer cells engineered to express the Tn antigen. These data suggest that Remab6 is specific for the Tn antigen and thus may be useful for detecting carcinomas or for targeted cancer therapy.

(155) Detection of N-glycans terminated with a3-mannose on a trans-Golgi glycosyltransferase and altered Golgi localization of a-mannosidase IA in advanced prostate cancer

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Biomarkers that can distinguish aggressive from indolent prostate cancer are needed to aid the treatment decision at the time of diagnosis. Recently, we have found that defected giantin in aggressive prostatic cancer cells is responsible for the alteration of Golgi targeting of glycosyltransferases and mucin O-glycans. But, its effect on N-glycans is not clear. Here, we report that in androgen-independent prostatic cancer DU145 and LNCaP C-81 cells, and androgen-sensitive prostate cancer LNCaP C-33 cells with depleted giantin but not LNCaP C-33 cells, α-mannosidase IA co-localizes with GM130/GRASP65 and a trans-Golgi enzyme GlcNAc: β4-galactosyltransferase 1 (β4GalT1) is decorated with N-glycans terminated with α3-mannose. The detection of these N-glycans on this enzyme in prostatic cancer cells with defected giantin is caused by shifted Golgi targeting of glycosyltransferases and α-mannosidase IA from giantin to GM130/GRASP65. Failure of α-mannosidase IA, mannosyl (α1,3)-glycoprotein: β2-N-acetylglucosaminyltransferase and α-mannosidase II to gain full access to the N-glycans on β4GalT1 under the dysregulated environment could explain how incompletely processed N-glycans are left on this enzyme in these prostate cancer cells. Further, in situ proximity ligation assays of α-mannosidase IA with GM130/GRASP65, and β4GalT1 with N-glycans terminated with α3-mannose are negative for LNCaP C-33 cells, normal prostate, and stage II prostatic tumor, and positive for LNCaP C-81 and DU145 cells, and stages III and IV prostatic tumors. Thus, the in situ proximity ligation assays based on altered localization of α-mannosidase 1A and detection of N-glycans terminated with α3-mannose on β4GalT1 may be used for distinguishing aggressive from indolent prostatic tumors. (Supported by Department of Veterans Affairs Merit Award 1I1 BX000985 and a State of Nebraska LB506 grant #2016-08).

(156) The action of recombinant lysosomal a-glucosidase (rhGAA) and amyloglucosidase on normal human and Pompe disease glycogen

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Following enzyme replacement therapy (ERT) in the mouse model of Pompe disease and in patient biopsies, it has been reported that detectable levels of muscle glycogen remain following treatment. To investigate this observation, experiments were initiated to evaluate the structure of glycogen isolated from normal and Pompe liver autopsy tissue to identify intermediates produced between glycogen and glucose, and determine if all glycogen could be completely degraded

following exposure to recombinant lysosomal α-glucosidase (rhGAA). Amyloglucosidase from *Aspergillus niger* was also used to investigate the degradation of the glycogens since the degradation of glycogen by amyloglucosidase is the method most often used to quantify glycogen. Intermediates detected during degradation by rhGAA include isomaltotriose, maltotetraose, maltose, isomaltose and maltotriose. Quantitatively, the major intermediates are isomaltotriose and maltotetraose. The abundance of isomaltotriose is interesting since it would be derived from glucose units with adjacent α-1,6 linkages which does not fit the commonly accepted model for glycogen structure. However, isomaltotriose has been reported in an acid hydrolyzate of glycogen. With respect to the degradation of glycogen neither rhGAA nor amyloglucosidase was capable of completely degrading the glycogen. This is due to the fact that the glycogen contains a core material which has residues other than glucose present. This core material contains about 40% protein and the carbohydrates include *scyllo*-inositol, *epi*-inositol, iditol, sorbitol, galactosamine, glucosamine, mannose, glucose and galactose. There are some slight differences in the relative amount of glucose between the residues from rhGAA and amyloglucosidase degradation. A very similar residue was observed on degradation of corn starch by the amyloglucosidase. These results indicate that additional research is warranted to further investigate if the structure of glycogen plays a role in the ability of rhGAA to degrade accumulated glycogen in the muscle tissue of Pompe patients following ERT.

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(157) Apical membrane expression of distinct sulfated glycans represents a novel marker of cholangiolocellular carcinoma

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Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver neoplasm, followed by hepatocellular carcinoma. ICC can be further subclassified as i) perihilar and ii) peripheral types, the latter histologically resembling small-sized intrahepatic bile ducts, such as interlobular bile ducts, cholangioles/ductules and the canals of Hering. Cholangiolocellular carcinoma (CoCC), now classified by the World Health Organization as a subtype of combined hepatocellular-cholangiocarcinoma, is currently regarded as a subtype of peripheral-type ICC. The present study was undertaken to determine whether sulfated glycans recognized by the MECA-79 monoclonal antibody could serve as a CoCC marker. Using immunohistochemistry, we show that MECA-79 sulfated glycans are preferentially expressed at the apical membrane of cholangiocytes found in small-sized intrahepatic bile ducts in normal liver and in canalicular structures formed in CoCC. We also report that apical membrane MECA-79 sulfated glycan expression colocalizes with that of mucin 1 (MUC1) core proteins. We also present immunoblotting of Chinese hamster ovary cells overexpressing FLAG-tagged MUC1 to show that MUC1 serves as a MECA-79 scaffold. Furthermore, we report that SSP-25 human ICC cells overexpressing *N*-acetylglucosamine-6-*O*-sulfotransferase 2 (GlcNAc6ST-2), but not GlcNAc6ST-1, exhibit membrane expression of MECA-79 sulfated glycans, suggesting that GlcNAc6ST-2 catalyzes MECA-79 epitope biosynthesis in cholangiocytes. Moreover, both wild-type and GlcNAc6ST-1 knockout mice exhibit apical membrane MECA-79 expression in small-sized intrahepatic bile ducts, namely interlobular bile ducts, while MECA-79 expression was completely absent in comparable tissues from GlcNAc6ST-1 and GlcNAc6ST-2 double knockout mice. These data collectively indicate that apical membrane localization of MUC1 proteins decorated with GlcNAc6ST-2-dependent MECA-79 sulfated glycans may mark cholangiocytes with cholangiolar/ductular differentiation and could serve as a useful CoCC marker.

(158) Alcohol effect on mucin O-glycosylation

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Alcohol abuse is known to affect N-glycosylation (Miller *et al.* Current Psychiatry, 5:80-7, 2004). But, its effect on mucin O-glycosylation is not clear. The goal of this study is to examine the effect of alcohol on mucin O-glycosylation. Air-liquid interface cultures of human bronchial epithelial (HBE) cellstreated for 72 h with30 or 80 mMethanolalong with untreated cells were used for the initial study. Paraffin sections of these HBE cells were stained with appropriate antibodies or lectins and then examined by immunofluorescence microscopy for Golgi morphology (giantin), key glycosyltransferases [Core 2 N-acetylglucosaminyltransferase (C2GnT)-1/L, Galβ3GlcNAc:α2,3sialyltransferase (ST3Gal1), and core 1 extension enzyme (β3GnT3)] and various O-glycan epitopes, including Tn (VVA), sialyl-Tn (antibody), T (PNA), sialyl-T (PNA after α2,3Neuraminidase treatment), sialyl Lewis a (antibody) and sialyl Lewis x (antibody). We found that the Golgi morphology and β3GnT3 were not appreciably affected by 30 or 80 mM ethanol treatment. However, there were ethanol concentration-dependent changes in the following parameters: C2GnT-1 was decreased and ST3Gal1 was increased. Tn, sialyl-Tn, T, 3'-sialyl-T, and sialyl Lewis a were increased while sialyl

Lewis x was decreased. Further, in addition to loss of C2GnT-1,Golgi fragmentation was also observed in HBE cells treated with 100 mM ethanol or 100 μM acetaldehyde, an ethanolmetabolite. These ethanol effects were prevented by co-treatment with 5 mMpyrazole, an alcohol dehydrogenase inhibitor, indicating that acetaldehyde is responsible for the ethanol effects. In addition, these ethanol and acetaldehyde effects were reversed by treatment with 35 μM blebbistatin, a non-muscle myosin IIA inhibitor which has been shown to restore Golgi morphology and Golgi targeting of C2GnT-1 in prostate cancer cells (Petrosyan *et al.* Mol Cancer Res19: 241-54, 2014). We conclude that alcohol abuse can cause alteration of mucin O-glycans by decreasing core 2-associated sialyl Lewis x and increasing core 1-associated sialyl Lewis a as well as short mucin O-glycans. The effects of altered mucin O-glycanscaused by alcohol abuse on mucus defense mechanism remain to be examined.

(159) O-GlcNAcase knockout disrupts mammalian cell autophagy

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The degradative process of autophagy ensures cellular homeostasis by recycling cellular constituents for energy production and degrading damaged proteins or organelles. Aberrant autophagy has been implicated in various pathological conditions including cancers as well as metabolic and neurodegenerative disorders.

Autophagy is regulated, in part, by glycans including the single monosaccharide posttranslational modification, O-linked N-acetyl-glucosamine (O-GlcNAc). Our previous work elucidated that loss of the enzymes required for O-GlcNAc addition (O-GlcNActransferase, OGT) or removal (O-GlcNAcase, OGA) leads to elevated induction of starvation-induced autophagy in the model organism C. elegans. In addition, several studies in C. elegans have noted that OGT is associated with phagosomes and protein O-GlcNAcylation influences autophagosome and lysosome fusion. We hypothesize that blocking O-GlcNAc removal from key autophagy-related proteins disrupts autophagy induction as well as autophagosome formation and fusion with the lysosome by disturbing proteostasis.

Key regulators of autophagy including BECN1 (involved in autophagy initiation) are known to be modified by O-GlcNAc. In this work, we studied proteostasis and autophagy induction by taking advantage of the defined genetics of newly derived Oga-/- mouse embryonic fibroblasts (MEFs, both primary and SV40-immortilized). We find that autophagy marker LC3 is elevated ~70% in Oga-/- MEFs compared to control cells by immunoblot. Immunofluorescence and flow cytometry data utilizing a danzylcadaverine reagent support these conclusions. Furthermore, we note that the levels of SQSTM1 (autophagosome cargo protein), BECN1, and VAMP8 (SNARE protein) are all elevated in Oga-/- MEFs. Together, these data support the hypothesis that disruption of O-GlcNAc cycling alters autophagic flux by increasing the stability of key components of the autophagic machinery. We suggest that regulating autophagy by modulating the O-GlcNAcylation of key proteins could be a powerful therapeutic tool.

(160) Proteomics reveals fatty acid synthase as a novel oxidative stress-induced interactor and inhibitor of the O-GlcNAcase

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O-linked-β-N-acetylglucosamine (O-GlcNAc) dynamically modifies and regulates thousands of nuclear, cytoplasmic, and mitochondrial proteins. Cellular stress, including oxidative stress, results in increased O-GlcNAcylation on numerous proteins and this is thought to promote cell survival. The mechanisms by which the O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA), the enzymes that add and remove O-GlcNAc respectively, are regulated leading to oxidative stress-induced changes in O-GlcNAcylation are not fully characterized.Here, we demonstrate that oxidative stress leads to elevated O-GlcNAc levels in U2OS cells, but has little impact on the activity of OGT. In contrast, the expression and activity of OGA are enhanced. We hypothesized that protein interactors of OGA may control the local activity or substrate targeting of this enzyme, resulting in stress-induced elevations of O-GlcNAc. We utilized the BioID proximity biotinylation technique in combination with Stable Isotope Labeling of Amino Acids in Cell culture (SILAC) to define the basal and oxidative stress-dependent interactome of OGA. Our study revealed 90 OGA-interacting partners, many of which exhibit increased binding upon oxidative stress. The associations of OGA with fatty acid synthase (FAS), filamin-A, heat shock cognate 70 kDa protein, and OGT were confirmed by co-immunoprecipitation. The pool of OGA bound to FAS demonstrates a substantial reduction in catalytic activity, suggesting that FAS is an inhibitor of OGA. Consistent with this observation, FAS overexpression augments stress-induced O-GlcNAcylation. Together, these data suggest that O-GlcNAcylation may be

one downstream effector of FAS that fine-tunes the cell's response to stress and injury.

(161) Enzymatic hydrolysis of pneumococcal type III polysaccharide

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In 1931, a soil-dwelling Bacillus was found to produce an enzyme capable of degrading the type III capsular polysaccharide (Pn3P) of Streptococcus pneumoniae (Spn). The expression of this enzyme was inducible in the presence of Pn3P, and the Bacillus strain was able to grow with Pn3P as the sole carbon source. A few years later, Sickles and Shaw were able to isolate the enzyme producing, Bacillus palustris (renamed later Bacillus circulans) strain from soil cultures. In several studies since, researchers have revisited this Pn3P degrading enzyme (Pn3Pd) while investigating Pn3P biosynthesis and its antigenic and immunological properties. Our lab has begun to investigate the structural mechanisms of glycoconjugate vaccine activation of adaptive immune responses against Pn3P. Our goal is to generate structurally defined vaccines that enrich for carbohydrate epitopes that are recognized by T cells, and therefore induce a stronger humoral immune response. For this purpose, we utilize Pn3Pd to generate Pn3 oligosaccharides for use in our structural and mechanistic study glycoconjugate constructs. In parallel, we investigate the potential use of this enzyme as a therapeutic for serotype III S. pneumoniae infections. The capsular polysaccharide is the major virulence factor for type III strains, as non-encapsulated mutant type III strains are completely avirulent. At present, we have isolated native Pn3Pd from B. circulans culture supernatant and characterized its activity and degradation products. We recently sequenced the genome of this bacterial strain and identified the gene of the enzyme through mass spectrometry-based proteomics. To investigate Pn3Pd as a potential therapeutic agent we assessed the ability of the enzyme to degrade the capsule on a live virulent type III Spn strain. Pn3Pd rapidly strips the capsule and disrupts Spn's ability to form biofilms in vitro. Future experiments will include in vitro lung epithelial cell adherence assays and in vivo colonization and lethal challenge.

(162) Truncated isoform of CD33 encoded by Alzheimer's disease protective allele is selectively diverted into an intracellular pool

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CD33 (Siglec-3) belongs to the Siglec family of I-type lectins, expressed widely on immune cells. There are two known splice variants of CD33- full length CD33M and a truncated CD33m variant that lacks the Ig-V-set-like sialic acid binding domain. Interaction between CD33M and sialylated ligands decreases phagocytosis by microglia in the brain, and negatively impacts clearance of amyloid plaques, a feature of Alzheimer's disease. In keeping with this notion, it is known that a human-specific derived *CD33* allele protective against Alzheimer's disease leads to increased splicing and decrease in expression of functional cell surface CD33M. It is assumed that the CD33m isoform also expressed on the cell surface and does not negatively regulate amyloid plaque clearance by the microglia. We hypothesized that protection against Alzheimer's could be due to diversion away from production of CD33M. Interestingly, we did not find truncated CD33m on the cell surface. Instead we found a large intracellular pool of CD33 mainly consisting of the truncated isoform, in neutrophils, monocytes and macrophages. The differential distribution is not due to lack of binding with sialic acid ligands, as sialidase treatment did not enhance CD33M endocytosis. Likewise treatment with different stimuli did not mobilize CD33m to the cell surface. Immunolocalization studies are being pursued to define where the CD33m product of the protective allele is localized.

"Gain-of-function" mutations that improve evolutionary fitness are well studied, as are "loss-of-function" mutations that facilitate escape from environmentally induced diseases like infections. The "Less is More" hypothesis suggests that "loss-of-function" mutations can also be adaptive for intrinsic fitness. Here we found a mechanism where the protection from disease is achieved via lowering levels of the functional ancestral CD33 molecule, which is thought to inhibit clearance of toxic amyloid plaques. To our knowledge this is the first example in which a "Less is More" mechanism operates by partially diverting mRNA towards a dysfunctional splice variant, thereby reducing production of an ancestral form that had become deleterious in the pathogenesis of a human neurodegenerative condition. This is also the first study showing the presence of an intracellular pool for any Siglec receptor.

(163) O-GlcNAc expression levels epigenetically regulate colon cancer tumorigenesis by affecting colon cancer stem cells via modulating expression of transcriptional factor MYBL1

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The post-translational modification of proteins by O-linked β-N-acetylglucosamine (O-GlcNAc) is a unique glycosylation of serine and/or threonine residues of a broad range of cytosolic and nuclear proteins and is catalyzed by a single enzyme, the O-linked N-acetylglucosamine transferase (OGT). Studies have implicated O-GlcNAc in the regulation of pathways important for oncogenic phenotypes, development, and epigenetic regulation of gene expression. We have investigated O-GlcNAc dependent phenotypes and epigenetic regulation of gene expression in colon cancer stem cells (CCSC). Xenograft tumors from colon tumor cells engineered to have lower OGT expression grew significantly slower than those formed from non-engineered cells, indicating OGT acts as a tumor suppressor in this model system. Significant reduction in the percentage of CCSC was observed in the tumor cell populations with OGT knockdown, compared to control cells, while tumor cells treated with TMG, a specific O-GlcNAcase (OGA) inhibitor showed increased levels of O-GlcNAc and an increased CCSC population, indicating that O-GlcNAc levels regulated the CCSC compartment. When grown in suspension, tumor cells with OGT knockdown showed a reduced ability to form tumorspheres (in both size and number), indicating a reduced self-renewal of CCSC due to reduced levels of O-GlcNAc. ChIP-seq experiments using an anti-O-GlcNAc antibody revealed significant chromatin enrichment of O-GlcNAc modified proteins at the MYBL1 promoter, which was also characterized by the presence of H3K27me3, repressive histone marks. Combining microarray and RNA-seq analyses, the transcription factor MYBL1 was observed to be increased in expression in tumor cells with OGT knockdown. Forced overexpression of MYBL1 in the tumor cells led to a reduced population of CCSC and reduced overall tumor growth in vivo, similar to the effects of OGT silencing. Moreover, two GC rich regions (CpG islands) near the TSS site of MYBL1 were identified, and their methylation status was regulated by O-GlcNAc levels. These results strongly argue that O-GlcNAc epigenetically regulates MYBL1 expression, functioning similarly to H3K27me3. Therefore, the aberrant CCSC compartment observed after modulating O-GlcNAc levels is likely to result, at least in part, from the epigenetic regulation of MYBL1 expression by O-GlcNAc modification, thereby significantly affecting tumor progression.

Key words: O-GlcNAc, Colon cancer stem cells, Epigenetic regulation, MYBL1, Methylation.

(164) Impaired lysosomal targeting leads to sustained activation of the Met receptor via ROS-dependent oxidative inactivation of receptor protein-tyrosine phosphatases

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The delivery of soluble acid hydrolases to lysosomes relies on a carbohydrate-dependent targeting mechanism in which mannose 6-phosphate (M6P) tags are added to the enzymes during their biosynthesis. These tags allow hydrolases to bind to specific receptors and traffic to the endolysosomal system. The enzyme GlcNAc-1-phosphotransferase (encoded by the GNPTAB and GNPTG genes) initiates the first step in the biosynthesis of the M6P tags. Loss of GlcNAc-1-phosphotransferase induces hypersecretion of hydrolases and causes profound lysosomal storage in cells. Little is known about how these cellular phenotypes affect the trafficking, stability and localization of cell surface glycoproteins. To address this question, we profiled the abundance of cell surface glycoproteins in WT and CRISPR-mediated GNPTAB-null HeLa cells. Selective exo-enzymatic labeling of these cells using ST6Gal1 followed by enrichment and proteomics identified changes in the abundance of numerous cell surface glycoproteins including reduced levels of the uptake receptor LRP1 and elevated abundance of multiple receptor tyrosine kinases (RTKs). Phospho-RTK array analysis further identified an increase in the activation of several RTKs including Met in the GNPTAB-null cells. Western blot analysis demonstrated greatly increased phosphorylation of Met in GNPTAB-null cells accompanied by higher steady-state levels of the receptor. Met signaling is regulated in part by dephosphorylation of specific tyrosines on the activated receptor by the action of receptor protein-tyrosine phosphatases (PTPs), such as Dep-1/PTPRJ. Our SEEL/proteomic findings showed that Dep-1 was less abundant on the cell surface of GNPTAB-null cells when compared to WT HeLa cells, suggesting GNPTAB-null cells may have less available Dep-1 to dephosphorylate phospho-Met. Further, lower concentrations of the PTP inhibitor, pervanadate, were needed to increase p-Met signal in GNPTAB-null cells compared to HeLa, indicating lower PTP activity. PTPs such as Dep-1 are enzymatically inactivated by oxidation of catalytic cysteines, when high levels of intracellular reactive oxygen species (ROS) are present. Our preliminary data shows that GNPTAB-null cells have increased ROS levels and that treatment of these cells with antioxidants partially reduces phospho-Met levels. Together, these findings highlight a novel mechanism whereby impaired lysosomal targeting might alter cell signaling – through ROS-mediated inactivation of protein phosphatases.

(165) Interactions of Mucins with the Tn or Sialyl Tn Cancer Antigens Including MUC1 are due to GalNAc - GalNAc Interactions

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The molecular mechanism(s) underlying the enhanced self-interactions of mucins possessing the Tn (GalNAc(α1-)Ser/Thr) or STn (NeuNAc(α2-6)GalNAc(α1-)Ser/Thr) cancer markers were investigated using optical tweezers (OT). The mucins examined included modified porcine submaxillary mucin containing the Tn epitope (Tn-PSM), ovine submaxillary mucin with the STn epitope (STn-OSM), and recombinant MUC1 analogs with either the Tn and STn epitope. OT experiments in which the mucins were immobilized onto polystyrene beads revealed identical self-interaction characteristics for all mucins. Identical binding strength and energy landscape characteristics were also observed for synthetic polymers displaying multiple GalNAc decorations. Polystyrene beads without immobilized mucins showed no self-interactions and also no interactions with mucin decorated polystyrene beads. Taken together, the experimental data suggest that in these molecules the GalNAc residue mediates interactions independent of the anchoring polymer backbone. Furthermore, GalNAc – GalNAc interactions appear to be responsible for self-interactions of mucins decorated with the STn epitope. Hence, Tn-MUC1 and STn-MUC1 undergo self-interactions mediated by the GalNAc residue in both epitopes, suggesting a possible molecular role in cancer. MUC1 possessing the T (Gal(β1-3)GalNAc-Ser/Thr) or ST antigen (NeuNAc(α2-3)Gal(β1-3)GalNAc-Ser/Thr) failed to show self-interactions. However, in the case of ST-MUC1, self-interactions were observed after sequential treatment of ST-MUC1 with the enzymes neuraminidase and β-galactosidase.

(166) Carbohydrate-mediated interactions between two thyroid cancer biomarkers and their reversible mutual sequestration

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Thyroglobulin (Tg) is the major glycoprotein produced by the thyroid glands. Tg plays central roles in thyroid hormone biosynthesis and in autoimmune thyroid diseases (AITD). The glycoprotein also serves as a pre- and post-operative biomarker of differentiated thyroid cancer (DTC). The endogenous lectin galectin-3 (Gal-3) has been shown to be another useful biomarker of DTC because Gal-3 is secreted by malignant thyroid cells. We hypothesized that the multiple complex-type glycans on Tg could serve as binding epitopes of Gal-3. As a result, these two biomarkers could potentially be involved in high affinity interactions. The objective of the study is to investigate the interaction between Tg and Gal-3 and discuss its potential consequences. Quantitative biochemical and biophysical studies showed that these two biomarkers produced by thyroid cancer cells interacted with each other with submicromolar affinity and formed insoluble complexes at their stoichiometric concentrations. One Tg molecule could bind up to 14 molecules of Gal-3. Such complex formation mutually sequestered both Tg and Gal-3, decreasing the concentration of their freely available forms in the solutions. Formation of the Tg–Gal-3 complexes was reversible as the preformed complexes were found to be sensitive to free Tg and free Gal-3. While free Tg rapidly dissolved preformed Tg–Gal-3 complexes in a concentration-dependent manner, Gal-3 was found to be much less efficient and slowly dissolved only a fraction of the preformed complexes at a relatively higher Gal-3 concentration. Complex formation between Tg and Gal-3 and the sensitivity of the complexes to free Tg and Gal-3 can potentially influence their biological functions. Interactions between Tg and Gal-3 might also interfere with their clinical detection, the same way Tg autoantibody (TgAb) is reported to introduce errors in Tg assays. The data presented here also support the notion that Gal-3 could potentially play a role in the homeostasis of Tg [1].

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(167) Structural characterization of the N-glycome from malignant melanoma cells reveals galectin ligands

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Glycan-galectin interactions play a vital role in cancer progression and metastasis. Recently, this sort of interactions have been shown to be critical in the glyco-pathogenesis of melanoma growth and migratory activity (1). Compared to the normal human epidermal melanocytes (NHEM) that they did not show any considerable ligands for galectin-1, human metastatic melanoma (MM) cells showed a high abundance of galectin-1 ligands. However, the glycan structures that define these glycan-galectin interactions are not well defined. Given the fact that galectin-1 and -3 are aberrantly expressed in MM, a comparative glycomics analysis of NHEMs and various MM cells was undertaken. The aim was to reveal any possible structural features that could explain the binding of galectin-1 and possibly other galectins to MM cells.

MALDI-TOF-MS analysis performed on NHEMs and MMs detected N-glycans that were mainly of complex type and core-fucosylated, and ranging from bi- to tetra-

antennary structures. On NHEMs, high mass range (*m/z* 5000-6000) N-glycans (on normal and α2-3 desialylated glycomic profiles) showed extended *N*-acetyllactosamine (LacNAcs) units that were predominantly "I"-branched, occasionally capped with NeuAcs. On the contrary, MM cells depicted high mass range (*m/z* 5000-8000) N-glycans that mainly consisted of linear LacNAc repeats with a minor contribution of "I"-branched LacNAc repeats, the level of which was dependent on the MM cell type. This in accordance with recent data showing that the "I"-branching β1,6 *N*-acetylglucosaminyltransferase 2, GCNT2, is decreased in MM compared with NHEMs. Desialylation studies showed that a considerable amount of glycans had α2-3 NeuAc residues. Therefore, the linear LacNAc repeats found on the MM cells were compatible with galectin-1 and -3 binding and MM cells are expected to present increase glycan-galectin interactions that would favor tumor growth and metastasis.

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(168) Identification of novel inhibitors of ppGalNacTs to target mucin secretion in asthma

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Asthma affects nearly 10% of children and 8% of adults in the United States. The three key pathologies of asthma are inflammation, bronchoconstriction and excess mucus secretion. Overproduction of mucus is also a pathological feature in other respiratory disorders such as chronic obstructive pulmonary disorder and cystic fibrosis. Current asthma therapies focus mainly on corticosteroids and adrenoceptor agonists to target inflammation and bronchoconstriction, and suitable therapies that target oversecretion of mucus are needed. Mucus consists primarily of gel-forming mucin polymers. Our approach to targeting mucus hypersecretion relies on high throughput screening (HTS) to identify novel inhibitors of glycosyltransferases involved in mucin biosynthesis. Here we describe two inhibitors identified from an initial HTS campaign. The screening utilized an unbiased mass spectrometry-based approach to identify potential inhibitors of the purified ppGalNAcT1 based on the ratio of unhydrolyzed UDP-GalNAc substrate to released UDP product. The HTS campaign identified 175 candidate inhibitors with Z-scores less than -3. These primary hits were then screened for the ability to inhibit secretion of the mucin MUC5AC from respiratory epithelial cells. Two candidate inhibitors displayed the desired activity and also caused reductions in MUC5AC transcript levels, presumably through a feedback mechanism. These compounds also caused reduced production of the Tn antigen in diverse cell lines, suggesting that they inhibit ppGalNAcTs in cells, as well as in vitro. The inhibitors were also characterized for their ability to inhibit ppGalNAcT1 in biochemical assays to determine the mechanism of enzyme inhibition. Furthermore, the inhibitors were evaluated using air-liquid interface culture of HBECs as an *in vitro* model for simulating the respiratory epithelium. Ongoing work includes further characterization of the candidate molecules for target specificity and minimal toxicity.

(169) Reduced molecular size and altered disaccharide composition of cerebral chondroitin sulfate upon Alzheimer's pathogenesis

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Alzheimer's disease (AD) is a progressive disorder leading to cognitive impairment and neuronal loss. Cerebral extracellular accumulation and deposition of amyloid ß plaques is a pathological hallmark of AD. Chondroitin sulfate (CS) is an extracellular component abundant in the brain. The structure of CS is heterogeneous with sulfation modification and elongation of the chain. The structural diversity of CS allows it to play various roles in the brain. Increasing evidence has shown that CS promotes aggregation of amyloid ß peptides into higher-order species such as insoluble amyloid ß fibrils. Difficulties in the structural analysis of brain CS, as well as its heterogeneity, limit the study of potential roles of CS in AD pathology. Here we utilized a microanalysis method with reversed-phase ion-pair high performance liquid chromatography and found that CS in the brains of Tg2576 AD model mice show a lower molecular size and an increased ratio of CS-B motif di-sulfated disaccharide. Our findings provide insight into the structural changes of cerebral CS upon Alzheimer's pathogenesis. We will also discuss CS in the brains of patients with AD.

(170) Changes in subcellular structure and ultrastructure of organelles in cultivated fibroblasts from the patients with congenital disorders of glycosylation

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Background: Congenital disorders of glycosylation (CDG) are a rare, clinically heterogeneous group of metabolic diseases caused by deficiencies of enzymes/proteins participating in glycosylation pathways. Up to this date, more than 100 of various CDG types (defined by the defective genes) have been described. However, the impact of the individual glycosylation defects on other cellular functions and its (ultra)structure is still not completely understood. The analyzed group: Patients with various glycosylation defects (of CDG type I or II, according to the sialylation pattern of

transferrin) were selected for the analysis. While in the CDG type I group (n = 3), the genetic defect had been identified (mutations were found in *PMM2*, *ALG8* and *RFT1* genes), the underlying molecular causes in the probands withCDG type II (n = 2) have not yet been elucidated. Material and methods: The experiments were performed in cultivated fibroblasts derived from skin biopsies from selected patients with CDG; three commercially purchased human fibroblast cell lines were used as healthy controls (passage <10). The level of reactive oxygen species (ROS) was detected by fluorescent probe dihydroethidium. Immunocytochemistry using giantin antibody was applied to visualize Golgi apparatus (GA). Using fluorescently labeled peanut agglutinin (PNA) lectin, global hyposialylation of mucin O-glycoproteins was studied. Alterations of cellular ultrastructure were analyzed by transmission electron miscroscopy (TEM). Results: All analyzed CDG patients showed markedly elevated levels of ROS in their fibroblasts, indicating the ongoing oxidative stress in the cells. In the CDG type II group, a distinct Golgi morphology was found (compared to both CDG type I and controls), characterized by its increased dilatation and/or fragmentation. Similarly, a typical finding for this CDG type was the increased fluorecent signal of the labeled PNA lectin, reflecting a higher ratio of hyposialylated mucin O-glycoproteins. Using electron microscopy, a dilatation of endoplasmic reticulum was observed in CDG type I patients, while the normal lamellar structure of GA (present in CDG type I) could not be detected in CDG type II. Conclusion: Subcellular analyses might help elucidate the pathophysiology of glycosylation disorders. Supported by: AZV16-31932A, RVO-VFN64165.

(171) Genetic glyco-engineering for improvement of biopharmaceuticals

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Glycotope's GEX™ platform comprises a comprehensive portfolio of proprietary glycoengineered human suspension cell lines. Gene editing technologies like the zink finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) or Crispr/Cas technology as well as classical overexpression techniques are very efficient tools to further gear up the glycosylation machinery for specific needs of human biopharmaceuticals.

Optimization of glycosylation can target the amount of e.g. fucose, galactose and sialic acid (NANA). As an example, the glycan influenced binding to liver based receptors like the asialoglycoprotein receptor is the key step to eliminate the molecule from the blood stream. As case study we show on the example of human factor VII expressed in a portfolio of different genetically modifies GEX cell lines the specific improvement of the glycosylation profile especially with regards to the reduction of liver receptor binding properties. Besides the generation of glycan with highest sialylation degree, the GalNAc moiety which exhibits high affinity towards the asialoglycoprotein receptor was removed by knockout of the respective transferases simultaneously on multiple alleles. Interestingly the knockout of the GalNT genes led to unexpected changes in other N-glycan features like antennarity of the N-glycans, sialylation degree as well as the amount of bisecting GlcNAc present. By these technologies a FVII molecule was generated which resembles to human plasma derived FVII to high extend.

(172) Assessment of glycosylation of recombinant HIV-1 envelope glycoproteins produced in a high-level protein expression system

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There is a critical need for development of an effective HIV-1 vaccine. The only partially successful trial RV144 pointed to the HIV-1 envelope glycoprotein (Env) as a viable vaccine candidate. Env is a trimer of gp120/41; the gp120 subunit is extensively glycosylated, with its *N*-glycans representing 50% of the total molecular mass. Env glycans are targets for many broadly neutralizing antibodies and, conversely, protect the virus from antibody neutralization by forming a glycan shield. Thus, Env glycosylation has to be considered in the design of future effective HIV-1 vaccines. Generally, the level of protein expression is important for vaccine production and therefore biotechnologically compatible cell lines, such as FreeStyle 293-F (293F), are often used. The 293F cell line can grow in suspension in serum-free media. In order to enhance protein production up to 10-fold, Expi293F cell line was developed. Such system would be useful for large-scale production of vaccine protein antigens. However, it is not known whether the elevated level of protein production affects protein glycosylation, either in general or in a site-specific manner. To test whether an enhanced level of expression affects protein glycosylation, we expressed recombinant HIV-1 gp120 (rgp120) using transiently transfected 293F and Expi293F cells. We generated rgp120 trimers from Env of a transmitted/founder virus WEAU, its naturally occurring immune-escape variant, and also generated variants with one or two *N*-glycosylation sites removed by site-directed mutagenesis. Next, we purified the recombinant glycoproteins and performed comparative glycosylation analyses. SDS-PAGE mobility assay and gel-shift mobility assay after treatment with Endo H did not reveal any apparent differences between the same rgp120 variants produced in 293F and Expi293F cells. However, we observed subtle differences in

glycosylation heterogeneity depending on the number of potential *N*-glycosylation sites. The ongoing experiments, including lectin and antibody recognition, monosaccharide compositional analysis, and a site-specific glycomic analysis will determine whether these two production systems differ in glycosylation of rgp120 variants and whether a higher glycoprotein expression can generate the appropriate vaccine antigens.

(173) Targeting binding of hypoglycosylated MUC1 to CIN85 to control tumor growth and prevent invasion and metastasis

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Overexpression and abnormal glycosylation of the epithelial glycoprotein MUC1 in cancer cells promotes metastasis. We have identified CIN85 (Cbl-interacting protein 85 KDa), as a binding partner of MUC1 in tumors. MUC1/CIN85 complex is found in early as well as advanced clinical stages of breast, ovarian, colon and prostate cancers among others. Co-localization of MUC1 and CIN85 on invadopodia enhances invasion and migration of cancer cells. Our hypothesis is that preventing the formation or dissociating existing CIN85/MUC1 complexes may result in a less aggressive tumor by decreasing local invasion, and preventing distant metastases. To test this hypothesis, we have already identified and tested two novel compounds that significantly reduce the association between hypoglycosylated form of MUC1 and CIN85, and at 10 μM concentration drastically reduce the migratory activity of mouse and human epithelial cancer cells. Ongoing experiments in *in vivo* mouse model will confirm the ability of these drug compounds to decrease tumor growth and metastasis. In particular, we are testing mouse tumor cell lines derived from ovarian tumors arising spontaneously in a MUC1 transgenic mouse and exhibiting different invasive and metastatic phenotypes. Our preliminary data also suggest that dissociation of the MUC1 and CIN85 complex decreases the expression level of hypoglycosylated MUC1, restoring the expression of its normal and fully glycosylated form. Localization studies performed so far detected CIN85 at several distinct cellular membranes and membrane-trafficking compartments. We are currently investigating the MUC1/CIN85-dependent signaling pathway with a particular focus on the ability of CIN85 to modulate MUC1 glycosylation by controlling its plasma membrane-Golgi trafficking. The data from this study will validate CIN85/MUC1 complex as a viable therapeutic target and support development and testing of more potent antagonists.

(174) The glycomics of Alzheimer's disease in human and mouse models

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Alzheimer's disease is characterized by significant neuronal degeneration and amyloid deposition within the brain. To investigate whether glycomic changes accompany and/or contribute to the pathology of the disease, we have begun to characterize the glycome of human and mouse models of the disease. We have analyzed glycosphingolipids, N-linked, and O-linked glycoprotein glycans of whole brains from an aggressive mouse model (5xFAD) and of patient-derived human induced pluripotent stem cells (iPS cells). Glycosylation was assessed in 5xFAD mice at 2.5 months of age, a timepoint at which significant plaque deposition has occurred but little memory deficit is detected. High-mannose, hybrid, and complex N-glycans were changed broadly in abundance in the 5xFAD mouse brains compared to wild-type. O-linked glycans were less significantly changed. Glycosphingolipids in the 5xFAD mouse were altered in regard to the abundance of specific glycan structures and in their ceramide heterogeneity. The glycome of iPS cells from Alzheimer's patients was largely unchanged in comparison to normal control iPS cells, indicating the importance of assessing glycosylation in cell types that are known to be affected in the disease. Differentiation of control and patient iPS cells toward neuronal lineages demonstrated that the Alzheimer's iPS cells were refractory to standard induction protocols. Further glycomic characterization of control and patient iPS cells over the course of neural differentiation will explore the importance of cell type specific glycosylation in neuronal function, survival, and differentiation.

(175) O-Fucosylation of Plasmodium falciparum proteins plays a key role in the malaria life cycle

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Plasmodium falciparum causes the most lethal form of malaria and is spread between humans by female *Anopheles* mosquitoes. Understanding the molecular basis of the transmission of this parasite is an important step towards developing measures to reduce the incidence of disease.

Several essential parasite adhesins expressed in the mosquito stages possess thrombospondin repeats (TSRs) – small protein domains that are conserved across different kingdoms of life. In metazoans, TSRs are O-fucosylated in the endoplasmic reticulum (ER) to ensure correct protein folding and secretion. Recently, glycoproteomics data on *P. falciparum* sporozoites revealed that the malaria parasite also O-fucosylates its TSR domains, a first for protozoans and the Apicomplexan parasites.

We have identified the *Plasmodium* O-fucosyltransferase responsible for glycosylation of TSR-possessing parasite adhesins. Targeted deletion of the encoding gene in *P.falciparum* revealed that it is essential for transmission from human to mosquitoes, and back again. Parasites lacking O-fucosylation are greatly attenuated for transmission, producing only a small number of oocysts within mosquitoes. Those that succeeded in producing oocysts yielded sporozoites with significant traversal, invasion and motility defects. We demonstrate that this is due to adhesin protein trafficking defects brought about by the absence of TSR O-fucosylation.

This is the first functional characterization of protein glycosylation machinery in a *Plasmodium* parasite. Our findings have important implications for malaria vaccine development, since the only vaccine approved to date (RTS, S) and other important candidate antigens (TRAP and CTRP) all contain TSR domains. It may also be relevant to the development of transmission-blocking drugs.

(176) Characterization and regulation of the functional O-mannose glycan on a-dystroglycan

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Over a dozen genes are essential for the proper O-mannosylation of the cell surface glycoprotein a-dystroglycan (a-DG), and this structure is the binding site for laminin globular (LG)-domain-containing extracellular matrix (ECM) proteins. Mutations in any of these genes result in multiple types of congenital/limb-girdle muscular dystrophies, can be associated with abnormal brain development, and are often silenced in cancer. Here, we further elucidate the O-mannose-initiated glycan structure on a-DG that is required for binding to LG-domain-containing ECM proteins. Using mass spectrometry, we show that the functional glycan contains a novel ribitol structure that links a phosphotrisaccharide to xylose. Additionally, we demonstrate that the *ISPD* (isoprenoid synthase domain-containing) gene product is a CDP-ribitol/-ribose pyrophosphorylase that presumably synthesizesthe reduced sugar nucleotide that serves as a donor for the insertion of ribitol in a phosphodiester linkage to a-DG. We also identify transmembrane protein 5 (TMEM5) as a xylosyl transferase that generates the substrate that is elaborated by ß4-glucuronyltransferase 1 (B4GAT1) and finally extended by the bifunctional a3-xylosyl-/ß3-glucuronyltransferases LARGE1 and LARGE2 which synthesize a glycosaminoglycan-like repeating disaccharide (-Xyla1,3-GlcAß1,3-) named *matriglycan*. Regulation of matriglycan length is currently unknown, however, overexpression of LARGE1 results in increased a-DG glycosylation, whereas sulfation, likely on a non-reducing end GlcA residue, by human natural killer-1 sulfotransferase (HNK-1ST) reduces levels of LARGE1-mediated a-DG glycosylation. Using mass spectrometry and NMR spectroscopy, we provide direct evidence that HNK-1ST catalyzes 3-O-sulfation of GlcA at the terminal, non-reducing end of matriglycan and blocks further extension by LARGE1. While a-DG from mouse heart and kidney are susceptible to ß-glucuronidase and a-xylosidase digestion of matriglycan, a-DG from brain (where HNK-1ST expression is greatest) is resistant to dual-glycosidase treatment, suggesting a possible cap on the glycan. We show that sulfated GlcA is resistant to ß-glucuronidase digestion and that removal of the sulfate cap facilitates glycosidase digestion. Taken together, we propose a novel structure – a ribitol in a phosphodiester linkage – for the moiety on which TMEM5, B4GAT1, and LARGE1/2 act to synthesize the functional receptor for LG-domain-containing ECM proteins and a brain, or tissue, specific mechanism in which HNK-1ST regulates matriglycan polymer length by non-reducing end GlcA sulfation.

(177) Loss and Gain of N-linked Glycosylation Sequons due to Variation in Cancer

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Years of sequence feature curation have culminated in a wealth of genomic and proteomic information, including known and predicted positions of variation and

glycosylation. Despite the availability of sequence feature data, there has been a lack of efforts to integrate this information with relevant supplemental data. We, therefore, set out to integrate non-synonymous single nucleotide variation (nsSNV) data from cancer samples based on the potential for altered glycosylation of affected proteins. In this study, we report the number and occupancy rate of currently known human N-linked glycosylation sequons (NLGs) and well-characterized glycoproteins. We then look at the effects of variation on these NLGs, and, finally, we investigate cancer-relatedness of glycosylation-impacting somatic variations by mapping all human NLGs to cancer variation data and reporting the resulting loss or gain of glycosylation sequon across cancer types. 15,314 of 20,199 human proteins in the Swiss-Prot database have at least one NLG, and all non-redundant NLGs were identified by three methods: extraction of annotated records, search by predictive software, and extensive string search for NXS/T motif. NLGs were both abolished and created by variations in as many as eight cancers. This work identifies candidate N-linked glycosylation-related cancer biomarkers that can be validated in future studies. We plan to connect these findings to a subsequent study looking at the role of differential expression of glycosyltransferases in cancer: we will look for overlapping genes and pathways in both studies to provide a more comprehensive understanding of N-glycosylation in cancer.

(178) Expression of fucosyltransferases is highly associated with metastasis of colorectal cancers

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In recent years, new approaches for early cancer diagnosis are earnestly needed, and some glycans have been identified as cancer biomarkers. Fucosylation has been associated with several malignant cancers, which is catalyzed by fucosyltransferases (FUTs). However, the pathological roles and regulatory mechanisms of their expression in colorectal cancer (CRC) remain unknown. In the current study, we addressed whether the expression of FUTs is up-regulated in CRC and correlated with tumor metastasis, disease recurrence, and poor survival in patients with CRC. In view of the heterogeneity of colon cancers, we initially studied samples from a homogenous group of patients with stage III colon cancers. Hierarchical cluster analysis segregated our cases into two main groups based on their glycogene expression, which was correlated well with whether the cancer recurred after surgical resection. The results from quantitative RT-PCR and immunohistochemical analyses indicate that FUT2, FUT4 and FUT8 are up-regulated in patients with recurrent CRC compared to those with non-recurrent CRC. This suggests that these enzymes might be associated with cancer recurrence. By using the fucose-binding lectin Ulex europaeus agglutinin I (UEA 1), we confirmed the higher levels of fucosylation in patients with recurrent CRC. Furthermore, bioinformatic analyses showed that there were highly different gene-expression profiles in recurrent and non-recurrent colorectal cancer tissues, including genes involved in cell junction and epithelial-mesenchymal transition. These results suggest fucosyltransferases may be useful prognostic indicators and therapeutic targets of CRC.

(179) A systems biology approach identifies FUT8 as a novel driver of melanoma metastasis

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Aberrant glycosylation has been previously associated with melanoma cancer progression based on studies that were mainly restricted to cell lines. Here, for the first time we have used a systematic, multi-disciplinary approach, using clinical melanoma primary and metastatic tissues to identify glycomic changes caused by glycosyltransferases (glycogenes) associated with melanoma metastasis. Specifically we show that upregulation of core fucosylation by increased FUT8 expression and downregulation of a-1,2 fucosylation by decreased FUT1 and FUT2 expression are features of melanoma metastatic tissues. Functionally, depletion of FUT8 reduces in vitro cell invasion without affecting cell proliferation. Moreover, in vivo FUT8 silencing strongly suppressed tumor dissemination and metastasis. We demonstrate that upstream, FUT8 is regulated by a transcription factor TGIF2. Finally, we show those downstream FUT8 targets are core fucosylated proteins involved in cell invasion, migration or metastasis such as L1CAM. Strikingly functions of L1CAM are core fucosylation dependent and alteration of its fucosylation result in altered cell invasion of melanoma cell lines. Thus, FUT8 and its targets represent

novel therapeutic targets for prevention or treatment of melanoma metastasis.

(180) Characterization of Expression of T-synthase (C1GALT1), Cosmc (C1GalT1C1), and Mucins in Tn-positive Colorectal Cancers

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The Tn neoantigen (GalNAca1-O-Ser/Thr) is commonly expressed in human cancers and is often related to tumor progression. In normal tissues, the Tn antigen is usually undetectable due to its conversion into extended O-glycans based on the Core 1 structure, by T-synthase, which requires the X-linked molecular chaperone Cosmc for correct folding and functioning. Studies in Tn-expressing cancer cell lines and pancreatic cancers have revealed loss of Cosmc expression in these samples due to either mutations or promoter hypermethylation. However, the mechanism(s) for Tn expression in many human cancers are largely unknown. In the present work, we characterized the expression of the Tn and sialylated Tn (STn) antigens, Cosmc and T-synthase at the transcript and protein levels, in paired human colorectal cancer (CRC) specimens. Using well-defined monoclonal antibodies, we confirmed prevalent Tn and STn expression in CRC samples. However, we did not observe mutations in the COSMC coding region. Instead, we observed increased expression of Cosmc and T-synthase at both mRNA and protein levels in the tumor samples when compared to their adjacent normal tissues. We observed a correlation between increased expression of T-synthase and elevated T-synthase enzyme activities in the Tn-expressing tumors, indicating that T-synthase was correctly folded and Cosmc is functional there. Moreover, while many tumors expressed both mucin 1 (MUC1) and mucin 2 (MUC2), their expression levels did not correlate with expression of the Tn neoantigen, indicating that mucin overproduction does not cause Tn antigen expression. The abnormal expression of large amounts of T-synthase in the tumors expressing the Tn antigen suggests alternative mechanisms that compromise activity of the T-synthase, including perhaps enzyme localization, or perhaps endogenous inhibitors of the enzyme. Thus, colorectal cancer differs from pancreatic cancer, since both commonly express Tn antigen, but likely differ in their mechanism for expression.

(181) Interactions of the Cytokine Pleiotrophin with Glycosaminoglycan and the PTPRZ Core Protein

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Pleiotrophin (PTN) is a glycosaminoglycan-binding cytokine involved in stimulating mitogenesis and angiogenesis. PTN is crucial in neural development and tissue regeneration, but is also consistently overexpressed in cancer cells. Although PTN is known to bind a number of different receptors, its interactions with the chondroitin sulfate proteoglycan receptor-type protein tyrosine phosphatase zeta (PTPRZ) are prominent in many aspects of its activities. Although it is generally accepted that PTN inhibits PTPRZ's phosphatase activity by inducing PTPRZ oligomerization, the details of the mechanism are not known. Previously, we have determined the structure of PTN in the absence of GAGs. In the present study, we conducted detailed investigation of PTN's interaction with both GAGs and the core protein of PTPRZ. We showed that PTN's specificity for GAGs is low and sulfation density of GAGs is the major determinant of binding affinity. In addition, the basic amino acid clusters in PTN do not act co-operatively when binding GAGs. Specifically, the N-terminal TSR domain (NTD) of PTN binds GAG oligosaccharides independently of the C-terminal TSR domain (CTD), allowing them to behave like separate domains and making PTN-induced receptor oligomerization through GAG crosslinking a possibility. However, FRAP experiments using membrane anchored pseudo proteoglycans showed that PTN is unable to cross link proteoglycans through their GAG chains alone, eliminating GAG-crosslinking as a possible mechanism for PTN-induced PTPRZ oligomerization. On the other hand, we have identified a segment of the PTPRZ core protein that has significant affinity for PTN and can act as an alternate PTN-binding site, raising the possibility that PTN-mediated PTPRZ oligomerization may involve both core protein and glycan components of PTPRZ. Experiments are now underway to investigate whether the new PTN-binding site in the core protein is necessary for PTPRZ oligomerization.

(182) Carbohydrate specific T cell stimulation by HIV envelope glycoprotein

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Since its outbreak about three decades ago, acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus-1 (HIV-1) has been a major threat to human health and a protective AIDS vaccine has not as yet been developed. The focus of past and current research has included structural and functional characterization of broadly neutralizing antibodies (bNAbs) isolated in HIV-1 infected individuals. Several of these bNAbs recognize the glycan shield of the HIV-1 envelope protein gp120. Hallmarks of T cell-mediated adaptive humoral immune responses are T and B cell proliferation and memory, antibody class switch, affinity maturation and secondary

(booster) immune response. Therefore, it is critical to recruit T cells to help induce high-affinity, long-lasting and protective antibody response against the gp120 glycan shield. This can only be achieved by investigating the cellular and molecular mechanisms of T cell activation by gp120. The goal of our project is to identify CD4+ T cell repertoires recognizing the glycan shield of HIV-1 envelope glycoprotein, gp120. We have gained evidence that glycans/glycopeptides presented on the surface of antigen presenting cells (APC) serving as potential epitopes to induce carbohydrate-specific T cells (Tcarb) response. Additionally, we demonstrate that certain populations of CD4+ T cells differentiate between glycosylated and non-glycosylated gp120 epitopes, suggesting the presence of carbohydrate recognition by CD4+ T cells. Using the discovery of such Tcarbs and their glycan epitopes, we can design and develop knowledge-based, new-generation HIV vaccines that will elicit strong and long lasting immune responses against HIV.

(183) Novel anti-Sialyl-Tn monoclonal antibodies and antibody drug conjugates (ADCs) target a cancer stem cell population and demonstrate in vitro and in vivo anti-tumor efficacy

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A successful therapeutic strategy for cancer will benefit from direct targeting of inherently chemoresistant tumor cells which are comprised in part of cancer stem cells (CSCs) that survive current cytotoxic treatment regimes and drive tumor resurgence. The sialyl-Tn (STn) antigen is a carbohydrate moiety present on bulk tumor and CSCs but rarely seen in normal adult tissue. Previous attempts to target this antigen in the clinic with synthetic glycan vaccines proved safe but lacked efficacy. Siamab's antibody therapy targeting STn is unique in its specificity, offering a best in class safety profile and has the ability to produce a more predictable therapeutic result than vaccines.

Siamab Therapeutics is developing highly specific humanized monoclonal antibodies and antibody drug conjugates (ADCs) targeting tumor-associated carbohydrate antigens, such as Sialyl-Tn (STn). Remarkable sequence homology across all anti-STn mAbs was observed in both heavy and light chains, and hot spots for hypermutation were identified. These antibodies were selected using our glycan microarray that enriches for candidates whose binding is protein-independent, highly specific and demonstrates exceptional target affinity. We utilized these anti-STn mAbs to assess the expression of STn and the known CSC marker CD133 in human ovarian cancer cell lines, xenograft models and patient primary carcinomas of the pancreas, colorectal and metastatic lesions. STn expression in these samples had a strong correlation with stem properties and CD133 expression. Common tumor lines (ovarian, stomach and breast) STn binding sites were determined per cell and antibody binding competition assays along with internalization studies were undertaken to choose lead candidates for antibody-drug conjugation (ADC) and subsequent *in vitro* and *in vivo* efficacy studies. Strikingly, in our OVCAR3 xenograft model 30 days after the last ADC dose was given, groups treated with the anti-STn ADC therapeutic exhibited mean tumor volumes below the Day 1 pre-treatment mean tumor volumes ($155mm^3$). Our humanized anti-STn ADCs decreased cell viability *in vitro* and reduced tumor volumes *in vivo*, suggesting that specific therapeutic targeting of STn in ovarian tumors may be an effective clinical strategy to eliminate quiescent CSCs.

(184) Application of the High-throughput GlycanMap® Platform to Discovery of Novel Glycomic Biomarkers

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Disease development and progression are often associated with alternations in glycosylation on tissue proteins and/or blood proteins. However, efforts to develop glycan-based therapies have been overshadowed by DNA- and protein-focused treatments becoming readily accessible. The recent development of new tools and techniques to study and produce structurally defined carbohydrates has spurred renewed interest in the therapeutic applications of glycans. Significantly different from nucleic acids and proteins in terms of biosynthesis, structures, and functions, glycans present in tissue/blood proteins would provide a valuable source of biomarkers to aid in the development of drugs, vaccines, and diagnostic tests, including companion diagnostics. In addition, glycomics holds potential for uncovering new therapeutic targets and mechanisms.

While glycosylation has been shown to be a critical component of many biological and cellular processes and has been clearly linked to diseases, exploration of glycans as a source of novel biomarkers and targets has traditionally been limited by the lack of practical, high-throughput analysis methods.

S-BIO's proprietary Glycan*Map*® platform can deliver both qualitative and quantitative data on *N*- and *O*-linked glycans, while providing both the throughput and repeatability required for robust biomarker studies. Glycan*Map*® analysis has been applied to the discovery of novel biomarkers of disease and drug treatment in multiple therapeutic areas, and it is compatible with many sample types including serum/plasma, urine, cerebrospinal fluid, and cell and tissue

lysates. The data shown here describes the application of this glycomics approach in both pre-clinical and clinical studies and demonstrates the utility of the Glycan*Map*® technology in biomarker discovery and development. In addition to evaluating individual glycans as novel biomarkers of disease and drug treatment, this approach also enables a more comprehensive analysis in which glycan changes can be analyzed with respect to the known glycan pathways. This approach provides valuable information on the underlying biology and reduces the inter-patient variability, facilitating smaller and more robust biomarker studies.

(185) Aberrant epigenetic regulation of glyco-genes and glycosylation related genes is involved in inflammatory diseases, diabetes and cancer

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Aberrant glycosylation is profoundly involved in virtually every complex disease including inflammatory and autoimmune diseases as well as cancer. Using epigenetic inhibitor of DNA methyltransferases, 5-aza-2'-deoxycytidine, on human cells in culture we have shown that many glyco-genes are regulated by DNA methylation and that their aberrant promoter methylation followed by aberrant expression has consequences on glyco-phenotype. In addition, by comparing DNA methylation and expression on several unrelated datasets representing different types of cancer we found the intersection of the glyco-genes with altered methylation/expression suggesting that epigenetic deregulation of glyco-genes is probably one of the most common way leading to aberrant glyco-phenotypes described in cancer. Our results also points to glyco-genes as a new important group of genes with changed expression in cancer through aberrant DNA methylation. We have shown as well that epigenetic deregulation of other glycosylation-related genes, such as transcription factors, has an effect on *N*-glycome composition and the disease outcome such as the case of HNF1A-MODY subtype of diabetes II. Our previous studies have shown the association between *HNF1A* promoter methylation and plasma *N*-glycome in blood of 840 patients with MODY, more precisely anntenary fucosylation and glycan branching were significantly decreased. We constructed CRISPR/Cas9-DNMT3A tool with aim to specifically change methylation at 4 CpG putative regulatory sites in the *HNF1A* gene and correlate this change with glyco-phenotype. In addition to glycomics we are also interested on glycoproteomic studies, focusing on the glycoprotein immunoglobulin G (IgG). The first genome wide association studies (GWAS) identified genetic loci associated with IgG glycosylation, some of which show pleiotropy with inflammatory bowel disease (IBD) and several other inflammatory diseases where IgG glycosylation changes have been reported. We were able to show that two of these GWAS loci, the glyco-gene *MGAT3* and *BACH2*, the transcription factor involved in B cell differentiation and maturation, were differentially methylated in the whole blood and B cells of IBD patients compared with healthy controls, and that these changes correlated with changes in IgG glycosylation observed in IBD – digalactosylation and sialylation. These new findings point on aberrant IgG glycosylation as one of the important element in IBD development and progression.

(186) Glycans Related to the CA19-9 Antigen Are Biomarkers of Pancreatic Cancer and Provide Added Value for Diagnostics

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The CA19-9 assay detects a glycan called sialyl-Lewis A (sLeA), a sialylated and fucosylated quatrasaccharide. It is the current best biomarker for pancreatic cancer but is not elevated in about 25% of patients using a cutoff that gives a 25% false-positive rate. We hypothesized that glycans related to sLeA also are biomarkers of pancreatic cancer and provide added value for diagnostics. We found two glycans that are independent biomarkers of pancreatic cancer. The first is a sialylated, non-fucoslated type-1 glycan (LSTa). Glycoforms of the proteins MUC5AC and MUC16 displaying LSTa were elevated in 64% (70/109) and 51% (55/109) of pancreatic cancers, respectively, at cutoffs giving 1% (1/91) false-positive detection of patients with pancreatitis or benign biliary obstruction. The second is sulfated and non-sulfated sialyl-Lewis X, an isomer of sialyl Lewis A. Mucin glycoforms displaying sLeX glycans were elevated in 52% (57/109) of pancreatic cancer patients at a 1% false-positive cutoff. Each of the three glycans—sLeA, LSTa, and sulfo-sLeX—is elevated in a distinct subgroup of patients, resulting in a panel of markers that performs better than CA19-9. The panel achieved 85% sensitivity (133/156 cancers) and 90% specificity (144/160 controls), giving a total accuracy of 88%, whereas CA19-9 achieved 54% sensitivity (84/156) and 86% specificity (138/160 controls) for a total accuracy of 70%. Furthermore, the cancer cells that produce the CA19-9 glycan have different locations and morphologies than those that express LSTa and sulfo/sialyl-Lewis X, suggesting that the glycans are markers of subtypes of cancer cell. These results raise the prospect of more accurate

diagnosis and prognosis using complementary glycans that arise from a distinct subtypes of cancer cells.

(187) Accurately Representing the Heterogneity of IgA1 O-glycosylation in patients with IgA Nephropathy

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Patients with several autoimmune disorders, chronic inflammatory diseases, and some infectious diseases exhibit abnormal glycosylation of serum immunoglobulins and other glycoproteins. The biological functions of these modifications in health and disease continue to be a significant area of interest in biomedical research. Specifically, the task of defining site-specific glycoprotein heterogeneity is recognized as an area that still needs a considerable amount of effort to fully understand the role of glycan heterogeneity in biological processes and disease pathogenesis. We have developed robust workflows for the analysis of the IgA1 clustered *O*-glycan heterogeneity in clinical samples from patients with a chronic kidney disease, IgA nephropathy (IGAN). IgAN is the leading cause of glomerulonephritis in the world with as many as 20-40% of patients progressing to end stage renal disease. Patients with IgA nephropathy have increased levels of nephritogenic circulating immune complexes that contain the immunoglobulin, IgA1. We and others have shown that IgA1 in patients with IgAN have altered *O*-glycan heterogeneity. This work demonstrates the progress we have made in characterizing the differing patterns of IgA1 *O*-glycan heterogeneity in patients with IGAN. IgA1 was isolated from serum of healthy controls and patients with IGAN, in order to determine each samples' specific *O*-glycosylation profile. Each patient's monomeric, polymeric, and circulating immune complex IgA1 were analyzed separately to determine if there was a difference in the glycan signature of the specific type of IgA1. The HR-MS Glycan Signature profile of both the IGAN patients and healthy controls was also tested using an existing lectin ELISA test for Gd-IgA1. The detailed characterization of glycoprotein site occupancy and glycan heterogeneity is required for a better understanding of the biological roles of individual glycoproteins and to determine the impact of the glycosylation on the proteins functionality. Our current results will demonstrate our ability to reliably provide quantitative comparison of individual sites of glycosylation across a range of *O*-linked glycosylation sites in order to determine a protein's Glycan Signature and how that signature relates to the proteins function. This work is supported by the NIH (GM098539).

(188) Elucidating the role of sialylation in cardiac function using a Drosophila model

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Drosophila heart has emerged as a powerful model system and a tool to study genes pertinent to the proper function and development of the human heart. Studies previously done in mammals have demonstrated the need for sialylation in the regulation of cardiac function. Aberrations in these post-translational modifications cause cardiac arrhythmias and defects in heart physiology by effecting large proteins such as voltage-gated ion channels. The *Drosophila* model provides a convenient *in vivo* system to examine the unknown genetic and molecular mechanisms of relevant cardiac genes collaborating with the sialylation pathway. This system is amenable to high throughput screening approaches at a low cost and can alleviate the ethical dilemmas often associated with vertebrate models. Importantly, this model provides key advantages for studying sialylation as *Drosophila* has substantially decreased genetic redundancy and complexity of neural glycosylation, is highly amenable to genetic manipulations, and provides opportunity for detailed analyses of heart physiology. Employing this system, we have analyzed knockout mutants of *Drosophila* sialyltransferase (*DSiaT*), the gene encoding the final enzyme in the sialylation pathway responsible for the transfer of sialic acid residues to glycoproteins. Our results suggest that sialylation mutants have cardiac abnormalities, including a reduced heart rate, commonly referred to as bradycardia. The mutant flies were also found to display cardiac arrhythmia phenotypes similar to those observed previously in mammals. Our current experiments are aimed at elucidating pathological mechanisms underlying the heart phenotypes. We are analyzing genetic interaction of *DSiaT* with candidate genes that potentially collaborate with the sialylation pathway in regulation of heart development and physiology. We hope that our project will contribute to better understanding of etiology of cardiovascular diseases, the top global cause of death in the human population. This project was support in part by the NIH/NS075534 grant to VP.

(189) Sweet and Stealthy Drug Delivery; Heparosan-based systems for enhancing therapeutics

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Many therapeutics need assistance with delivery to be most effective in the patient.Poly[ethylene glycol] (PEG) polymers are widely used by pharma to enhance the physical, chemical, and/or biological nature of promising drug candidates. PEGylation has resulted in several $Billion/year drugs. PEGylation protects the cargo when in the body and prolongs therapeutic action. For patients, these attributes translate to fewer injections and side-effects. However, PEG's tissue accumulation, and rising occurrence of immunogenicity (~25% of the naïve population) are liabilities. Therefore, pharma is interested in PEG alternatives for next-generation medicines.

The University of Oklahoma andCaissonBiotech LLC, an Oklahoma biotech company, are collaborating to develop and commercialize a platform technology, HEPtune™, to add heparosan polymer to therapeutic cargo or secondary delivery platforms (e.g., liposomes, micelles). Heparosan is a natural "self" polysaccharide that is the biosynthetic precursor for heparin, a widely used drug, and heparin sulfate (HS), an extracellular glycan found on most cell types. We were inspired by the approach of certain pathogenic bacteria, *Pasteurella multocida* Type D and *Escherichia coli* K5, that employ heparosan coatings to evade host defenses and not generate an immune response. Furthermore, sulfation is required for the known biological activities of heparin/HS (e.g., coagulation and growth factor systems), the uptake by the HARE clearance receptor, and the cleavage by heparanase. Therefore, we predicted that unsulfated heparosan polymers would be 'ignored' in the extracellular spaces thus should circulate in an intact form for a prolonged period in the bloodstream. Our patented chemoenzymatic technology harnesses the *Pasteurella* heparosan synthase (PmHS1) to synthesize sugar polymers with both very narrow size distribution and defined chemical activation that facilitates selective heparosan coupling to therapeutics or delivery platforms.

In experiments in rodents and primates, HEPtune™ extends drug pharmacokinetic half-life in the bloodstream. This sugar-based delivery system has some superior attributes over PEGylation including new intellectual property, natural degradation pathways (thus no detrimental tissue accumulation), and lack of immunogenicity.

(190) Knocking-out fdl gene in a baculovirus host insect cell line using new CRISPR-Cas9 tools for lepidopteran insect cell lines

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The baculovirus-insect cell system (BICS) has been widely used to produce recombinant proteins for basic research and is being used to produce several biologics approved for use in human or veterinary medicine. One drawback of the BICS is its limited *N*-glycan processing capability compared to mammalian expression systems. Fused lobes (FDL) is an *N*-glycan specific *N*-acetylglucosaminidase that trims the terminal α1,3-branch GlcNAc from *N*-glycans and antagonizes their elongation in insect cells. In this study, we aimed to knock-out the *fdl* gene in insect cell lines used as hosts in the BICS, including Sf9 and High Five™, by CRISPR-Cas9 technology. We found existing CRISPR-Cas9 tools used to edit insect and insect cell line genomes failed to induce detectable indels in Sf9 cells. Therefore, we used various insect U6 promoters to construct new CRISPR-Cas9 vectors and assessed their utility for site-specific genome editing in these cells. We discovered surprisingly tight and unpredictable cross-species restrictions, which dictated the ability of various insect U6 promoters to support CRISPR-Cas9 editing in cell lines from different insect species. Ultimately, we created new CRISPR-Cas9 vectors containing U6 promoters that supported editing in both host cell lines of interest. We also discovered a novel lepidopteran insect-specific sequence element required for optimal U6 promoter function. Finally, we successfully isolated SfFDLKO cell lines by knocking-out Sf-*fdl* gene in Sf9 cells with the CRISPR-Cas9 vector. Knocking-out Sf-*fdl* dramatically reduced the production of a paucimannosidic *N*-glycan structure, $Man_3GlcNAc_2$, and increased the production of extended *N*-glycan structures, $GlcNAcMan_3GlcNAc_2$ and $GlcNAc_2Man_3GlcNAc_2$, as previously observed in *fdl*-KO Drosophila melanogaster cell lines. Our new CRISPR-Cas9 tools can be used for host cell engineering in the BICS and our newly-isolated SfFDLKO cells will facilitate our future bottom-up glycoengineering efforts.

(191) Comprehensive Glycoproteomics of Glioblastoma Biospecimens

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Introduction: The extracellular matrix (ECM) constitutes 10-20% of brain volume and provides a microenvironment for maintaining the essential functions of central nervous system. Over 80% of ECM proteins are glycosylated; however, little is known about the glycosylation pattern for brain ECM proteins. Here we present a sensitive platform to analyze N-glycoproteome and O-glycoproteome for brain ECM glycoproteins. Method: Human brain glioblastoma biospecimen tissue microarray slides were analyzed. The slides were de-waxed and re-hydrated in water. Trypsin was added to the surface of tissue section for overnight digestion. The released peptides were either enriched with hydrophilic interaction liquid chromatography (HILIC) or strong anion exchange stage tips. Half of the HILIC-enriched peptides were analyzed with C18 LC-MS/MS at Thermo-Fisher Q Exactive Plus mass spectrometer. N-glycans were released from the remaining half of the HILIC-enriched peptides with PNGaseF and analyzed using a Agilent 6520 Q-TOF mass spectrometer coupled with nano-fluidic HILIC liquid chromatography. Chondroitinase ABC and heparin lyase enzymes were used to digest chondroitin sulfate (CS) and heparan sulfate (HS) chains, respectively, from peptides enriched using strong anion exchange cartridges. Preliminary data: Brain ECM components are organized into three principal compartments: the basement membrane, the perineuronal nets and the neural interstitial matrix. Both perineuronal nets and the neural interstitial matrix comprise a dense network of hyaluronan, tenascins, link proteins, and proteoglycans. Proteoglycans are composed of a core protein to which one or multiple glycosaminoglycan (GAG) side chains attach. Chondroitin sulfate proteoglycan (CSPG) and heparan sulfate proteoglycan (HSPG) are the two major families.

We observed high levels of ECM glycoproteins from surface digestion on histological tissue sections, including CSPGs/HSPGs (brevican, versican, neurocan, CSPG4,

CSPG5, and perlecan), tenascins (tenascin and tenascin R), and hyaluronan and proteoglycan link protein 1. Our data identified site specific glycosylation on ECM glycoproteins from brain. We used CAD-based tandem mass spectrometry of enriched N- and O-glycopeptides to identify peptide sequences and glycan compositions. We used the in-hous GlycoReSoft software for automated data analysis for N-glycoproteome and O-glycoproteome. Taking the versican CSPG as an example, we mapped multiple un-reported N-glycosylated, mucin type O-glycosylated, and GAG-linked sites in the core protein. We made a detailed comparison for N-glycoproteome and O-glycoproteome for human glioblastoma biospecimens and tumor-adjacent normal samples to understand the dynamic alterations of ECM glycosylation during cancer progression.

(192) Highly sensitive detection of fucosylated glycans with a novel click chemistry probe

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Disease-associated changes in glycans are often observed. This glycan alteration has been considered to be good candidates for biomarkers of various diseases. Particularly, fucosylated glycans and their biosynthetic enzymes have been reported to be upregulated in various types of cancers including lung cancer, and the aberrantly high levels of core-fucosylated glycans are correlated with poor clinical outcomes of non-small cell lung cancer (Honma *et al.*, *Oncology*, 2015, 88, 298-308). This suggests that the detection of increased fucosylated glycans derived from lung cancer cells could lead to development of a novel biomarker for lung cancer, which could contribute to improvement of lung cancer therapy. However, the current techniques for the detection of fucosylated glycans are not sufficient in specificity and sensitivity, which has hindered our developing novel fucose-related biomarkers.

In this study, using a chemical biology approach in combination with click chemistry, we developed a new highly sensitive chemical probe for fucosylated glycans. We newly synthesized 4 novel fucose analogs having an alkyne group which is utilized for click chemistry-based glycan labeling and detection. By in vitro fucosyltransferase assays, we found that one of the fucose analogs, "7-alkynyl-fucose", is the best substrate among the fucose analogs for all 5 enzymes tested (Fut1, 2, 4, 8, 9). Accordingly, when added to cultured cells, 7-alkynyl-fucose can label and detect cellular glycans with higher sensitivity than existing probes. In addition, 7-alkynyl-fucose did not show an obvious cytotoxicity which was observed for 6-azide-fucose. Furthermore, our MS-based structural analyses confirmed that 7-alkynyl-fucose was correctly converted to the corresponding GDP-form in the cells and incorporated mainly into the core position of N-glycans. Consistent with this, mutant cells deficient for the core-fucosylation enzyme, Fut8, showed a dramatic decrease in signals of labeled glycans with 7-alkynyl-fucose. These results suggest that our new fucose probe is a powerful tool for the detection of fucosylated glycans, especially core fucose, with high sensitivity, which could be applied to glycan detection and biomarker discovery.

Keywords: Click chemistry, Fucose, Fucosyltransferase, Glycan detection

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(193) Homogenous detection of glycosyltransferase activities with universal bioluminescent assays

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Glycosyltransferases (GTs) play a pivotal role in many biological processes and attracted the interest of scientists in diverse therapeutic areas, including diabetes, and infectious diseases. Because of the importance of this class of enzymes, there is a need for biochemical assays to monitor their activity, their mode of regulation, and to search for selective and potent activity modulators. Traditional assays for GT activity analysis are not easily configured for rapid GT activity detection because they rely on cumbersome detection technologies such as radiometric and antibody-based assays. In a typical glycosyltransferase reaction, after sugar transfer from the donor nucleotide-sugar substrate, the nucleotide moiety is released as the reaction product. Therefore, an assay that detects the nucleotide molecule could be generically used to assess all glycosyltransferases activity in vitro. We developed four bioluminescent assays for measuring GT activities based on UDP, GDP, UMP and CMP detection. Each of these assays is performed in one-step detection that relies on converting simultaneously the nucleotide product to ATP and the latter into light in a luciferase reaction. The light output is proportional to the nucleotide concentration produced ranging from low nM to 25-50μM which is a reflection of enzyme activity. These assays are highly sensitive, and robust. Various applications of these assays will be presented, including studies on specificity of transfer of different sugars to different acceptors by various glycosyltransferases such as B4GALT1 and MGAT3. These assays were shown to be adequate for determining enzyme kinetics parameters such as Km for donor and acceptor substrates. We

demonstrate that this generic assay platform can be used to characterize different GTs from different groups such as fucosyltransferases sialyltransferases and phosphoglycosyltransferases. We show their utility in screening for specific GT inhibitors and the study of their mode of action. The development of these bioluminescent nucleotide detection assays (UDP-Glo, GDP-Glo and UMP/CMP-Glo) will enable the investigation of a large number of GTs and may have significant impact on diverse areas of Glycobiology research.

(194) Cellular O-glycome Reporter/Amplification to explore O-glycans of living cells

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Glycans are founds in all organisms, regulate cell signaling, cell adhesion, and host physiology, and contribute to disease when altered. However, current technologies to analyze glycans, using chemical or enzymatic glycan release coupled to mass spectrometry (MS), are limited due to poor sensitivity and a need for a relatively large amount of sample material. Mucin-type O-glycans are a major class of cell surface and secreted glycans, present on >80% of proteins that traverse the secretory apparatus, that are especially challenging to analyze due a lack of enzymes to release these structures. To address this, we devised a strategy, termed Cellular O-Glycome Reporter/Amplification (CORA), to bypass glycan release and amplify the O-glycome directly from cultured cells. We incubated cells with a peracetylated chemical O-glycan precursor (Ac_3GalNAc-Benzyl) that crosses the plasma membrane, gets de-esterified by cytosolic esterases, is taken up into the Golgi apparatus, and is modified by native glycoysltransferases using native nucleotide sugar donors as it traverses the secretory apparatus. Bn-O-glycans representing the cellular O-glycome are secreted into the culture media for easy purification and MS analysis. We show that this approach increases sensitivity ~100 – 1000-fold and works in a variety of primary and cancer cell types from different organisms. In total, we analyzed ~20 different cells and identified ~80 unique compositions, including some novel structures, and coupled with computational modeling were able to estimate the size and complexity of the human cellular O-glycome. Thus CORA offers an approach to amplify the glycome and investigate the role of glycans in health and disease.

(195) Carbohydrate microarray as a new technique to rapidly detect Sallmonella

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An increasing number of human and animals around the world suffered from salmonella-caused diseases during the last decade. As one of the main food-borne pathogens of both human and animals, salmonella has to be detected with a rapid method with higher sensitivity, specificity and excellent repetition.Since the FimH protein, which is located in the structure of the type 1 fimbriaeof salmonella, can specific bind to D-mannose residues, carbohydrate microarray may be one of the valuable tools to detect salmonella, which hasmany advantages such as low amount of samples required, high specificity and high throughput screening.

Herein, we investigated the application of carbohydrate microarrays in salmonella detection. First, different kinds of carbohydrate microarrays were prepared and the the detection conditions of the microarray was optimized. The carbohydrate microarray prepared by Man-1and NHS showed the best binding efficiency with salmonella strain ATCC31685. Different concentrations of carbohydrates or bacteria were tested. The minimum used concentration of carbohydrate is 313 μmol/Land the minimum detectable concentration of the bacteria is 106cells/mL. The Man-1/NHSmicroarray can distinguish two kinds of salmonella strains(ATCC31685 and ATCC9184), which possess different affinity towards mannosidic structure.The nondestructive nature of carbohydrate microarray allows the detected salmonella to be harvested and tested for antibacterial susceptibility. Thesensitivity and specificity of this carbohydrate microarray will be further improved. And thecarbohydrate microarray will bedevelopedfor screening FimH protein antagonists.

Keywords: salmonella, carbohydrate, carbohydrate microarray, D-mannose, FimH, antagonist

(196) Semantic Web Technologies for Integrating Glycan-related Databases in GlyTouCan

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Semantic Web technologies allow life science databases to be stored and queried directly over the web, thus allowing such data to be traversed computationally. Since semantics are defined by ontologies, data on the Semantic Web can be used for inferences, such that unexpected relationships between data can be found by using inference algorithms. In terms of glycoscience data, related information, such as

protein and lipid information that pertain to a particular glycan can be potentially retrieved online semi-automatically.

We have been developing an international glycan structure repository called GlyTouCan (http://glytoucan.org) using Semantic Web technologies which would enable the integration of various informatics resources. GlyTouCan is a freely available, uncurated registry for glycan structures that assigns globally unique accession numbers to any glycan independent of the level of information provided by the experimental method used to identify the structure(s). That is, any glycan structure, ranging in resolution from monosaccharide composition to fully defined structures, including glycosidic linkage configuration, can be registered as long as there are no inconsistencies in the structure [1].

GlyTouCan is fully based on Semantic Web technologies and currently provides links to other major glycan databases such as GlycoEpitope, the bacterial, plant and fungal carbohydrate structure database CSDB, GlycomeDB and UniCarbDB. Links are now also available to other databases such as PubChem and PDB. Users can register their own glycan structures in GlyTouCan and thus retrieve links to other databases containing the given structure.

The glycan structure representation called WURCS (http://wurcs-wg.org) is used as the main format for storing glycans in GlyTouCan, ensuring uniqueness of even ambiguous glycan structures while representing them as linear text strings. This allows for 1) efficient searching of the repository for existing structures because a simple text comparison can be used and 2) the use of WURCS in URIs to link directly to the detailed information of the glycan structure.

The MIRAGE project (http://www.beilstein-institut.de/en/projects/mirage) also recommends that GlyTouCan accession numbers be used when reporting glycomics experiments that include identified glycan structures. Thus, in the future, GlyTouCan may serve as a portal to search for glycan-related publications as well as other related information.

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(197) Detection of post-translational modification of cancer biomarkers via proximity ligation assay

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The detection of proteins released in the bloodstream can be a valuable tool for diagnostic, prognostic and follow-up therapy of pathological conditions. Additionally, it has been discovered that defects in post-translational modifications (PTMs) of proteins can lead to development of numerous disorders and human disease. Thus, the detection of biomarkers with disease specific PTMs would be an important tool for detection of human maladies. However, despite their biological importance the detection of PTMs is hindered by the limited methods available to identify them. In the attempt to circumvent this issue, we have applied solid-phase proximity ligation assay (sp-PLA) for the detection of PTMs in specific cancer biomarkers. This assay depends on the recognition of the target molecule by three affinity binders. These binders were polyclonal antibodies against the target protein, which were used for both capture and detection, along with antibodies, or lectins, to a specific PTM of interest. The assay was compared with sandwich ELISAs for detection of glycosylation of CD44 and E-Cadherin, and phosphorylation of p53 and EGFR. The assay was found to have superior dynamic range, and limit of detection compared to the ELISAs as it was able to detect the targets in concentrations as low as 10fM. Furthermore, sp-PLA demonstrated great performance in plasma, and serum samples. Thus, we have established the use of sp-PLA as an apt method for the detection of biomarkers and their respective PTMs, which offers a more sensitive and specific detection system for diagnosis and prognosis of the biomarker's corresponding disease.

(198) Systematic Quantification of Human Cell Surface Glycoprotein Dynamics

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Glycoproteins on the cell surface are essential for cells to interact with the extracellular matrix, communicate with other cells, and respond to environmental cues. Although surface glycoproteins can dramatically impact cell properties and represent different cellular statuses, global and site-specific analysis of glycoproteins only on the cell surface is extraordinarily challenging. An effective method integrating metabolic labeling, click chemistry and mass spectrometry-based proteomics was developed to globally analyze human cell surface glycoproteins. Surface glycoproteins metabolically labeled with a functional group were specifically tagged through copper-free click chemistry, which is ideal because it is quick, specific and occurs under physiological conditions. Sequentially, tagged glycoproteins were enriched for site-specific identification by mass spectrometry. Systematic and quantitative analysis of the surface N-sialoglycoproteome in cancer cells with distinctive invasiveness demonstrated many N-sialoglycoproteins up-regulated in invasive cells, the majority of which contained cell adhesion-related domains.

One of the most important roles of glycans is to protect proteins from degradation, but systematic investigation of the effect of glycans on protein degradation has yet to be performed, including for important surface glycoproteins. Combining the current method with multiplexed proteomics, we systematically analyzed surface glycoprotein dynamics and measured their

half-lives. Glycosylation sites located outside of any domain had a notably longer median half-life than those within domains, which strongly suggested that glycans within domains regulated protein interactions with other molecules while those outside of domains played a role in protecting protein from degradation. Furthermore, surface glycoproteins with catalytic activities were much more stable, compared with those with binding and receptor activities. This method allows us to globally and site-specifically analyze glycoproteins on the cell surface, and will have extensive applications in the biological and biomedical research communities.

(199) GRITS Toolbox - A freely available software system for processing and archiving of glycomics data

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In recent years the technical advances in mass spectrometry (MS) have led to a steady improvement in accuracy and throughput of MS-based glycomics analysis. In addition the increased recognition of glycan importance in biological processes and disease has created growing interest in the study these molecules and their interaction with other molecules. These trends necessitate the development of sophisticated software systems to process, organize and annotate the produced MS data.

GRITS Toolbox is an extendable software system that has been developed for processing, interpreting and archiving of glycomics MS data. The program allows loading of MS data from different types of instruments into the software using mzML or mzXML file format. Once loaded, the integrated data interpretation module, called Glycomics Elucidation and Annotation Tool (GELATO), can annotate the MS data with glycans and glycan fragments. An extensive set of graphical user interfaces can then be used to visualize, review, modify and export the annotated data. In addition, the annotations from different experiments can be displayed side by side and compared to identify differences in the glycosylation patterns between the analyzed samples. For the annotation of the MS data GRITS Toolbox uses glycan structures from curated databases of Mammalia glycans. However users also have the options to create their own databases and use these for the annotation of MS data. New databases can be created by retrieving structures from web databases, drawing new structures in GlycoWorkbench or a combination of both. This enables users to create customized databases beyond the Mammalia scope of the database integrated in GRITS Toolbox.

To allow the creation of a comprehensive archive of the performed experiments, GRITS Toolbox also includes optional software modules to enter, browse, modify and store metadata and other associated data. These modules allow describing the project, the analyzed samples, and the experimental protocols used for the analysis. In addition, supplementary files, such as reports, notes, papers or other experimental data, can be archived together with the metadata and the MS data.

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

(200) Detection of Antibody Inhibition of Influenza H5N1 Binding to a Sialoglycan Receptor Using Surface Plasmon Resonance (SPR) and its Use as a Neutralizing Antibody Screening Assay

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Highly pathogenic avian influenza A H5N1 continues to be a public health problem with 163 cases (including 47 deaths) reported in Egypt between November 2014 and April 2015 to WHO. Hyperimmune globulin therapy is currently being used for several infectious agents such as Rabies, Hepatitis B, Varicella-Zoster, and Tetanus. Vaccinia and Anthrax Immune Globulins have also been approved in the US. An anti-H5N1 Equine F(ab')2 product has recently obtained EMA Orphan Drug status. In order to screen individual plasma donations for high neutralizing titers to pool into a hyperimmune product, a fast and reliable neutralizing antibody assay is needed. Currently, the hemagluttination inhibition (HAI) and microneutralization (MN) assays are used to detect neutralizing antibodies against Influenza viruses; however, each assay requires the use of live virus. We developed a Surface Plasmon Resonance (SPR) assay to measure anti-H5 HA antibodies for neutralizing activity which has no requirement for live virus. Briefly, biotinylated multimeric glycans containing sialic acid moieties were attached to a streptavidin coated surface and acted as a model for influenza cell-surface receptors. Properly folded multimeric H5 HA recombinant protein micelles preincubated with a dilution sequence of antibodies or serum were then injected over the glycans. The results of the antibody dilutions preincubated with H5 HA were compared to H5 HA only, and an IC50 was calculated. Using the IC50 measurement we could rank the mAb and polyclonal sera in order of neutralizing activity. We have also used this assay to measure neutralizing antibody to other flu strains. This SPR assay may also be adapted to measure the neutralizing antibody against other infectious agents where the host receptor is known.

(201) Novel Designer Microarray Approach to Pinpoint Epithelial O-Glycans as Ligands: Application to Rotaviruses

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Mucins are glycoproteins widely distributed on epithelial and endothelial cells, leukocytes and in secretions. They harbor enormously diverse populations of O-glycans, among which are ligands for adhesive proteins of infective agents, endogenous recognition proteins and antigens that are developmentally-regulated or cancer-associated. The detection and isolation of the O-glycan determinants for structural characterization from among hundreds of glycans within O-glycomes can be a formidable task.

We have addressed the need for a streamlined, microscale method to pinpoint and characterize glycan ligands among highly heterogeneous O-glycans. The method involves robotic arraying of the O-glycans from mucins as fluorescent lipid-linked conjugates, neoglycolipid [1,2], concomitantly with resolution by multi-dimensional chromatographies, monitoring by microarray analysis and mass spectrometry followed by micro-immuno-sequencing.

In this presentation, using porcine stomach mucin as a model, we demonstrate the potential of the new approach by applying to the characterization of O-glycan ligands for the cell adhesion proteins VP8* of two rotaviruses P[10] and P[19] that mediate the initial steps of the viral infection. Enteroviruses of this family are a major health concern worldwide among infants and children under the age of five [3], and are also pathogenic to animals including pigs [4]. Rotaviruses are known to bind to high molecular weight epithelial glycoproteins, mucins [5], but the glycan sequences bound on such mucins have not yet been characterized to our knowledge. Identification of the ligands present in epithelial tissues involved in the initial steps of rotavirus infection is important for our understanding of viral tropism and future therapeutic designs.

This designer array approach for the pinpointing and characterization of ligands among hundreds of glycans on an epithelial mucin O-glycome is a significant advance over existing methods.

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(202) Automated Analysis of Bacterial Peptidoglycan Structure

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Peptidoglycan (PG) is an essential component of the bacterial cell envelope. This macromolecule consists of glycan chains alternating N-acetylglucosamine and N-acetylmuramic acid, crosslinked by short peptides containing nonstandard amino acids. Structural analysis of PG usually involves enzymatic digestion of glycan strands, separation of disaccharide-peptides by reversed-phase HPLC followed by collection of individual peaks for MALDI-TOF and/or tandem mass spectrometry. Here, we report a novel strategy using shotgun proteomics techniques for a systematic and unbiased structural analysis of PG using high-resolution mass spectrometry and automated analysis of HCD and ETD fragmentation spectra with the Byonic software. Using the PG of the nosocomial pathogen *Clostridium difficile* as a proof of concept, we show that this high-throughput approach allows the identification of all PG monomers and dimers previously described, leaving only disambiguation of 3-3 and 4-3 crosslinking as a manual step. Our analysis confirms previous findings that *C. difficile* peptidoglycans include mainly deacetylated N-acetylglucosamine residues and 3-3 crosslinks. The analysis also revealed a number of low-abundance muropeptides with amino acid substitutions in the C-terminal position and peptide sequences not previously reported.

(203) Glycosyltransferase Bump-hole Engineering to Dissect O-GalNAc Glycosites in Living Cells

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O-GalNAc glycosylation partakes in important physiological processes,[1] and aberrant O-GalNAc glycans are tightly linked to tumor malignancy.[2] Efforts to delineate the context of this glycosylation type are complicated by the existence of >20 human glycosyltransferases that introduce the first, peptide-proximal monosaccharide GalNAc, the polypeptide GalNAc transferases (ppGalNAcTs).[3] Despite partial peptide substrate redundancy, altered expression of single ppGalNAcTs is associated with disease, calling for a method to deconvolute the functions of discrete isoenzymes. Herein, progress toward "bump-hole" engineering of ppGalNAcTs is presented. Thereby, a "hole" is engineered by mutagenesis into the active site of a ppGalNAcT, making the enzyme compatible with a "bump" present in an unnatural UDP-sugar substrate. A traceable chemical handle present in the bumped substrate allows for the specific detection of glycoproteins elaborated by the engineered enzyme by bioorthogonal ligation.[4] The establishment of a cell-based bump-hole system requires the manipulation and fine-tuning of UDP-sugar biosynthesis to facilitate substrate delivery. Uncovering the native substrate profiles of individual ppGalNAcTs will shed light on their roles in disease and eventually reveal novel cancer biomarkers.

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(204) A Toolkit for Interactive and Batch Analysis of Glycomics and Glycoproteomics Mass Spectrometry Data

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The analysis of mass spectrometry data sets involves a series of signal preprocessing steps required before making assignments of putative biological identities to observed patterns. Ideally, these steps would be consistent for any experiment, and generic enough to be applied regardless of the type of glycoconjugates studied. In practice, methods are specific to different glycoconjugate classes. For proteomics projects, there exist mature tools for carrying out necessary data transformations; however, these methods are largely inappropriate for or incapable of handling the diversity of glycomics and glycoproteomics.

We present a collection of data structures, data access procedures, algorithms and software components that streamline analyses of glycan and glycopeptide high throughput liquid chromatography-mass spectral data. These components can be used interactively through a Python interpreter or Jupyter Notebook session, or combined into programs to analyze large batches of data. To demonstrate the value of these components, we present a series of case studies showcasing the utility of these tools:

- Retrieve structure information from GlyTouCan or Glycome-DB to construct a search space for glycans, constrained by motif or substructure, taxonomic class, and precision.
- Interactively investigate a mass spectrum to investigate whether a set of compositions are present based upon charge state deconvolution, isotopic pattern fitting and adduct formation.
- Process a full glycan LC-MS dataset using the constructed database, identifying and quantifying glycan compositions.
- Incorporate the identified glycans from the previous LC-MS analysis into a glycopeptide database to search against an LC-MS/MS run derived from the same sample tissue to identify site-specific glycosylation.
- Visualize the results of each analysis, summarizing the abundances of different features of the data.

(205) Towards automated identification of glycan branching patterns using multistage mass spectrometry with intelligent precursor selection

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Glycans play important roles in a wide range of biological processes and their activities are closely related to their fine structure details. Unlike proteins, which are consisted of simple linear chains of amino acid, branching is a unique feature of glycan sequence, making high-throughput and high-sensitivity analysis of glycan structures extremely challenging.

Tandem mass spectrometry with collision-induced dissociation (MS^2) has been used successfully in glycan sequence determination [1]. Glycosidic bond cleavage with B-/Y- and C-/Z-ions affords sequence and branching pattern information while cross-ring fragmentation with A-/X-type ions may occur, although frequently of low abundances, to indicate linkage positions [2]. However, MS^2 is not always sufficient and multiple-stage mass spectrometry (MS^n, $n > 2$) is frequently required to provide detailed structural information.

The major difficulty for successful application of MS^n and thereafter automated high-throughput glycan structural identification is precursor-ion selection. Unlike MS^2, sequential MS^n requires careful selection of fragment ions as precursors for the next stage of product-ion scanning. Currently, the widely-used strategies for precursor-ion selection are either manually by experienced experts or simply picking-up the most intense peaks. Manual selection requires considerable expertise and time, whereas product-ion spectrum generated by the most intense peak may not be structurally informative. The concept of 'intelligent precursor selection' to guide MS^n experiments has been described [3] but a systematic approach for achieving is lacking.

In this communication, we describe an intelligent precursor-ion selection (GIPS) strategy for glycan identification. The GIPS program consists of two key components: a hierarchical Bayesian model to calculate *likelihood* of each candidate glycan and a statistical model to derive the '*distinguishing Power*' of a specific fragment ion to allow selecting structurally most informative ions as precursors to guide multistage MS^n scanning for accurate glycan identification. Using various different types of glycan molecules, we demonstrate how the GIPS approach makes the assignment of branching patterns of glycans using MS^n, and that compared with the conventional method the GIPS generally requires fewer rounds of product-ion scanning. The proposed approach represents a major step forward towards high throughput and automated glycan omics.

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(206) Development of a tool for extracting common glycan patterns recognized by avian influenza A virus.

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The purpose of our study is the elucidation of glycan recognition mechanisms through the use of glycoinformatics techniques. Due to the advancement of glycomics technologies, many experimental results are being accumulated from mass spectrum data, microarray data, glycan array data, etc. Information of the experimental data related to glycans have been published in databases that integrate glycan information such as KEGG GLYCAN [1], the Consortium for Functional Glycomics (CFG) [2], Glycosciences.de and JCGGDB[3]. Because glycan structures have a complex structure, and since tools that can analyze these experimental data are few, manual analysis is very time-consuming. Therefore, we have developed a web tool called MCAW (Multiple Carbohydrate Alignment with Weights) for the extraction of binding recognition patterns from glycan affinity data. The MCAW algorithm is based on the KCaM [4] algorithm, which aligns pairs of carbohydrate structures, and ClustalW [5], which is a popular multiple amino acid sequence alignment algorithm. The MCAW tool has now been implemented on the web as a part of RINGS [6] to output a multiple glycan alignment of a set of glycan structures. In the tool execution result, alignment results are illustrated as a figure, and percentage of each monosaccharide and the glycosidic bond are represented. Presently in the MCAW tool, we have confirmed the possibility of aligning up to 123 glycans at once. We have analyzed several data sets of glycan structures from binding affinity data which we obtained from the CFG (Consortium of Functional Glycomics). As an example, we performed an alignment of high-affinity glycan structures from glycan array data of mutant human HA influenza A virus. As a result, sialic acid a2-3 was most highly aligned. We were able to confirm this result of the MCAW tool with the report of Dortmans [7].

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

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

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

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

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

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

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

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

(208) Carbohydrate Structure Notation Directed Towards Interdisciplinary Cooperation

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In the field of life science such as glycomics, proteomics, metabolomics and lipidomics, saccharide derivatives are used as research targets or tools. The carbohydrate derivatives such as glycoproteins, glycolipids are composed of components of sub-structures. These information of molecular structure are stored in databases of the respective field in various formats. In recent years, using databases have become essential to conduct research, however, it is not easy process to obtain information about data of required compounds. One approach is database cooperation to utilize a molecular structure as a key. Recently, The use of the Semantic Web technology is being considered in various fields. Our group develops the Web3 Unique Representation of Carbohydrate Structures (WURCS) utilizes the Semantic Web technology [1]. And also we are developing various tools utilizing WURCS. Therefore, we have developed a new notation for representing carbohydrate derivatives directed towards the Semantic Web based on components of sub-structures.

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(209) The Utility of IdeZ Protease in Glycan Profiling of Therapeutic Antibodies

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Characterization of glycans on therapeutic IgGs is critical as stability, half-life, and clinical efficacy are affected by the glycoforms present on the molecule. In addition, the primary structure of the antibody along with post-translational modifications must be carefully analyzed to ensure the integrity of the product. Mass spectrometry is the most commonly used method to analyze these biological therapeutics but the molecular weight of an intact mAb make this a challenge.

We describe here the cloning and characterization of a highly specific antibody protease from *Streptococcus equi ssp. equi*, IdeZ. IdeZ cleaves under native conditions at one single site below the hinge region, yielding a $F(ab')_2$ fragment and 2 Fc fragments. Treatment of the $F(ab')_2$ with a reducing agent yields smaller molecules; 2 Fd' and 2 LC. These fragments are approximately 25 KDa each, allowing for analysis by mass spectrometry.

We have compared the specificity of this enzyme to another antibody protease, IdeS, from *Streptococcus pyogenes*. These enzymes have very similar specificities, the exception being that IdeZ is able to cleave murine IgG2a more efficiently than IdeS. We have used IdeZ treated antibodies and protein A magnetic beads to create separate pools of Fc and Fab fragments. These substrates are then treated with Rapid PNGase F to release glycans which are subsequently PROC labeled for LC/MS analysis. Using this method, we are able to accurately assign specific glycoforms to either the Fab or Fc fragment of the antibody.

(210) RAIDR– A Rapid Method for the Microextraction of O-Glycans

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O-glycosylation, a common post-translational modification of serine and threonine residues, regulates biological processes through direct interactions of glycans with binding proteins. O-glycosylation modulates protein stability, conformation, solubility, and lifespan; however, it has also been implicated in the pathogenesis of a number of diseases, which has stimulated interest in analytical methods capable of isolating O-glycans for characterization. Here, we report a method termed Rapid Ammonium hydroxide Isobutyric acid O-glycan Deglycosylation Reaction (RAIDR) for the microscale extraction of O-glycans for glycomic characterization. When carried out with the assistance of microwave radiation, the efficient O-glycan release was achieved in 15 minutes representing the most rapid method reported to date. The selectivity of the RAIDR method was evaluated using bovine fetuin, a glycoprotein with both O-glycan and complex N-glycan structures. Following RAIDR release, protein/glycoprotein was separated from released oligosaccharides and subjected to treatment with PNGase F, resulting in two pools of oligosaccharides that were permethylated and analyzed using MALDI-TOF-MS and LC-ESI-MS. The results indicated less

than 1% of total N-glycans were liberated during RAIDR release, demonstrating the selectivity of the method for O-glycans. When the RAIDR method was applied for the analysis of human milk, peaks representative of the full complement of Bile Salt Stimulating Lipase (BSSL) heterogeneous O-glycans were observed. Additionally, direct comparison with traditional β-elimination indicated that RAIDR release is more efficient. Subsequent experiments involving the analysis of human blood serum support the conclusion that the RAIDR protocol represents a rapid, inexpensive and selective method for the microextraction of O-glycans.

(211) Characterizing Glycosylated Proteins and Their Interactions Using Sparse-Labeling NMR

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The carbohydrates (glycans) that cover the surfaces of most mammalian cells act as a first line of communication with other cells and the large number of regulatory proteins that control growth, migration, and defense against invading organisms. Structural characterization of the interactions between glycans and cell-surface proteins is obviously important in the development of agents that can intervene in any of these processes. Characterization using conventional structural techniques can, however, be a challenge. Most cell-surface signaling proteins are themselves glycosylated. Native glycosylation, which is often required for function, is highly complex and heterogeneous. This works against crystallization for X-ray studies, and it limits the use of uniform isotopic labeling and deuteration for NMR studies. We will describe an alternate NMR procedure that is based on sparse labeling with single or small subsets of isotopically labeled amino acids, and illustrate its application to terminal domains from the axon guidance protein, Robo1, and the receptor protein tyrosine phosphatase, LAR.

The sparse labeling approach is based on the fact that structures for individual domains from proteins in their non-glycosylated forms are often known from prior X-ray or NMR work. The issues to be resolved are typically inter-domain interactions and domain interactions with specific glycans, in this case heparan sulfate (HS) oligomers. NMR information collected from sparsely labeled protein residues, including chemical shift perturbation, residual dipolar coupling (RDC) and long-range effects from paramagnetic tags is often adequate to address these issues. Utilization of this information does, however, require assignment of sparsely labeled sites, something that cannot be done using traditional triple resonance methods. A new genetic algorithm based approach has been introduced to overcome this obstacle.

Robo1 and LAR provide ideal illustrations of the methodology. They normally carry large N-glycans that can affect both domain orientation and HS binding. HS binding is known to modulate signals controlling cell migration and synapse formation. Studying the interaction of synthetic HS fragments with two domain fragments from these proteins, we have been able to identify binding sites, and demonstrate through modeling, how HS fragments could modulate interactions with other proteins and modulate signaling.

(212) NIST Interlaboratory Study on Glycosylation Analysis: Variety and Variability of Methods

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Changes in glycosylation may significantly modify the biological activity of monoclonal antibodies. Thus, analysis of their glycoforms is essential, whether it is batch-to-batch analysis of a drug candidate, comparison of the glycan profile of a biosimilar, or complete glycomics profiling of a new drug. There are several published methods to quantify and identify glycoforms in proteins, but there are only a handful of multi-lab studies to assess the performance of these various approaches.

Consequently, an interlaboratory study was conducted by NIST to determine measurement variability in identifying and quantifying N-glycans across laboratories. This work describes results from a recent study on glycosylation analysis of a NIST reference material, NISTmAb, from 103 reports worldwide.

Each laboratory was asked to perform glycosylation analysis of two monoclonal antibody samples, NISTmAb and a glycan-modified NISTmAb, using their chosen method. A pilot interlaboratory study consisting of a core group of experienced laboratories was conducted prior to the final study.

103 reports were submitted by 73 laboratories from Europe (42%), North America (38%), Asia (18%), and Australia (2%). Reports came from industry (43%), university (35%), research (9%), government (9%), standard setting organization (3%) and hospital (1%) laboratories.

About three-fourths of the reports analyzed enzyme-released glycans (74%), 20% glycopeptides, 2% intact protein, and 1% protein fragments. Glycans were derivatized by fluorescent labelling, permethylation, reduction, and ethyl esterification. To determine the identity of the glycans, labs used accurate mass (45%), retention time, MS/MS, migration time, exoglycosidase, and MSn. Peak area was used as quantification method in 68% of the reports, followed by MS1 intensities, summation of isotope peaks, peak height, and isotopic dilution.

Data analysis is in progress. Preliminary Youden plots of the three abundant glycans G0F, G1F, and G2F showed that 87%, 85%, and 69% of the normalized laboratory measurements, respectively, are consistent with the robust consensus values at an approximate 99% confidence level.

Determining variability of monoclonal antibody glycosylation profiles from a huge number of laboratories and range of measurement methods provides a baseline for comparison in the rapidly developing field of glycosylation analysis.

We thank all the scientists who participated in this study.

(213) Cholera Toxin subunit B binding to heterogeneous gangliosides on cell mimicking surfaces

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Prior studies have reported that Cholera Toxin subunit B (CTB) binding to cell membrane surfaces did not correlate to the amount of its preferred binding receptor, GM1. It was hypothesized that CTB binding specificity and avidity were modulated by the cooperative action between heterogeneous ganglioside receptors. To better understand the essential nature of binding cooperativity in a hetero-multivalent binding mechanism, quantitative analysis of multivalent membrane recruitment onto the cellular surface is critical. We have developed a nanocube sensor coupled with complex reaction analysis to quantitatively explore the multivalent binding mechanism. The nanocube sensor is surrounded by a lipid bilayer that possesses the same physical and chemical properties as cell membranes. This novel sensor is an ideal tool for studying binding cooperativities because receptors can freely diffuse and rotate on 2D fluidic cellular membranes, allowing receptor self-organization to enable multivalent interactions.

We recently studied pentameric CTB binding to mixtures of gangliosides. Interestingly, very weak binding gangliosides like GM2 could be activated by strong binding gangliosides (e.g. GM1, fucosyl-GM1, and GD1b) resulting in up to a ten-fold higher CTB binding capacity. However, the cooperative action between GM3 and other receptors was negligible. We demonstrated that allosteric regulation is not the major cause of the observed binding enhancement. We hypothesize that the increase of CTB binding is caused by a reaction rate enhancement mechanism, "reduction of dimensionality". After CTB attaches to the first ganglioside, subsequent binding events are confined on the 2D membrane surface. Therefore, the weak GM2 receptor could participate in second or higher binding events because its surface reaction rate was at least 10^4 times higher than the rate in bulk solution. This hypothesis was verified by altering the surface reaction rate. Our results indicate that the traditional ligand-receptor binding assay that detects protein interactions with only one specific receptor at a time (e.g. microarray technology) is not appropriate to explore multivalent interactions. In order to discover all possible gangliosides participating in the binding process, we designed a new membrane perturbation protocol that can efficiently screen possible ganglioside receptors involved in CTB binding.

(214) Characterizing glycoproteins using EndoH/PNGaseF in combination with high-resolution accurate-mass (HRAM) mass spectrometry

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We present here the methodology for enriching and characterizing glycoproteins and glycopeptides. Taking advantages of strong-anion exchange (SAX) chromatography, endoglycosidase H, PNGaseF, and the advanced mass spectrometry, we demonstrate the improved enrichment of glycopeptides, more comprehensive quantitative studies of glycan heterogeneity, as well as the elucidation of glycopeptide topologies from purified proteins to complex mixtures.

(215) Bacteriophage receptor binding proteins as carbohydrate specific diagnostics and therapeutics

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Bacteriophages are considered the most abundant and potentially the most assorted life forms on earth. Phage receptor binding proteins (RBPs) allow phages to specifically target their hosts and consequently represent a diverse reservoir of bacterial targeting proteins. In recent years, several new technologies for the detection of pathogens have been developed using the ability of RBPs to be species- or even strain-specific. We have demonstrated that recombinant phage RBPs are readily expressed in bacteria or plants and several of these proteins can reduce bacterial growth *in vitro* and/or colonization *in vivo*. Recently, we have developed high throughput techniques for RBP identification and new RBP-based diagnostic platforms.

Bacteriophage RBPs are often difficult to identify based on homology due to their considerable diversity. We established an RBP discovery assay using phage genome expression libraries and high-throughput screens to identify binding partners that recognize the host bacterium. Screening libraries expressing the proteomes of the *Salmonella* Typhimurium phage P22 and the *Escherichia coli* O157:H7 typing phage 1 (ECTP1) confirmed that Gp9 is the sole RBP in P22, previously shown to recognize bacterial lipopolysaccharide (LPS) O-antigens. We also identified Gp145 as a new ECTP1RBP. Using ELISA assays and far Western blots, we show that Gp145 binds to *E. coli* O157:H7 and does not recognize the other *E. coli* strains tested. Interestingly, Gp145 does not bind to the LPS O-antigen, so we are currently trying to identify the *E. coli* receptor.

Since RBPs bind to bacteria with high affinity, this trait can be exploited in a diagnostics-based platform. We fused the *Thermotoga maritima* cellulose binding module, CBM9, to Gp9 and Gp145. This module binds to β-D-Glcp-(1-4)-β-D-Glcp and is commonly used to enable protein binding to paper. When this tag is attached to the N-termini of Gp9 and Gp145, the sensitivity of the paper-based platforms is much higher than when using the RBPs alone. The pathogens could also be detected in other matrices such as chicken cecal contents and chicken juices. Taken together, these results show that RBPs represent an exciting new technology for microbial detection and treatment that are inexpensive, easy to use and readily scalable.

(216) Domain specific N-glycan profiling of a Fc-fusion antibody

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Antibody N-glycosylation is a PTM that has a tremendous influence on the efficiency and immunogenicity of an antibody. A comprehensive analysis of the N-glycosylation profile of biopharmaceuticals and biosimilars is therefore crucial in the development and production of protein based drugs and the characterization of N-glycosylation is a key regulatory requirement according to ICHQ6B guidelines.

N-glycosylation of widely used IgG1 biopharmaceuticals exclusively occurs at a particular site of the Fc domain. The different N-glycans attached to that site are typically quantified after fluorescence-labeling by hydrophilic interaction liquid chromatography, HILIC. New generation therapeutics consisting of Fc-fusion proteins show N-glycosylation at both the Fc domain and the fusion domain of the molecule. Therefore, differential monitoring of glycosylation of both domains is required.

In that study we present a strategy generating domain-specific and quantitative data on the N-glycosylation profile of Fc-fusion therapeutics. Using the FragITkitTM tool (Genovis) with the enzyme IdeS coupled to agarose beads, the target is specifically cleaved into both domains. Separation of these domains and N-glycan release by PNGaseF generates domain-specific glycan pools. These are finally labelled by 2-AB and analyzed by HILIC and fluorescence detection.

For the Fc-fusion protein investigated obtained 2AB-glycan pools were initially analyzed by coupling HILIC to mass spectrometry. This setup generates data on retention time and molecular weight allowing to securely identify the N-glycans present in the sample. In further routine or high throughput analyses only HILIC fluorescence detection is performed as the N-glycans can be matched via their retention time pattern to the exemplary mass spectrometry data. In this approach, HILIC allows sensitive, efficient and quantitative glycan profiling.

We used these techniques to elucidate the domain specific glycosylation profile of a Fc-fusion antibody. This revealed mainly fucosylated asialo-glycans for the Fc domain whereas the fusion partner showed high levels of sialylation

To put it in a nutshell, we are demonstrating a technique for efficient and quantitative domain specific N-glycosylation profiling for antibodies and Fc-fusion mAbs which allows for example fast batch to batch consistency testing, release testing of new drugs or the establishment of a RMP corridor for the development of biosimilars.

(217) Methods for determining ganglioside distributions in lipid rafts

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Lipid rafts are dynamic membrane microdomains with distinct lipid and protein compositions. They are involved in regulation of membrane functions and serve as signaling platforms of the cell. There is an increasing interest in the investigation of lipid raft dynamics and their precise compositions due to the established role of lipid rafts in pathogenesis of numerous human disorders.

Various approaches to isolating lipid rafts have been used to separate them from the bulk (non-raft) membranes. Most often, detergent-resistant membranes (DRMs) enriched in cholesterol and sphingomyelin and thought to be related to rafts, are extracted using the non-ionic detergent Triton X-100 (Tx-100). However, Tx-100 causes a major redistribution of membrane gangliosides and is therefore unacceptable as a detergent for lipid raft isolation for studying gangliosides and ganglioside-associated proteins. Published non-detergent methods for lipid raft isolation were not sufficiently effective or reproducible in our hands. In this work lipid rafts were isolated from bulk lipids by density centrifugation after membrane solubilization using a detergent that does not result in redistribution of gangliosides in membranes: Brij O20 (previously known as Brij 98). Successful raft isolation was confirmed by Western blot analysis of the lipid raft marker protein flotilin and the non-lipid raft membrane protein transferrin receptor and the method was found to be highly reproducible.

Since the aim of this work is to develop a method for ganglioside purification from isolated lipid rafts for subsequent analytical studies, several methods were then tested to solubilize and separate gangliosides from excess of Brij O20. Brij O20 is polyoxyethylene ether with a molecular weight of 1150 Da (similar size to gangliosides), and readilly forms micelles (including mixed micelles with gangliosides). We employed several purification techniques, including organic solvent extraction, ion-exchange chromatography and

adsorption-partition chromatography. The outcome was assessed by HPTLC. Organic solvent extraction and ion-exchange chromatography were not efficient enough in separating Brij O20 from gangliosides. Better separation was achieved by adsorption-partition chromatography, however further optimization is needed. This work will enable more detailed lipid raft analysis in respect to ganglioside composition and structure and lead to improved resolution of lipid-protein relations within lipid rafts.

(218) A rapid sample preparation and high throughput analysis of N-glycans by magnetic bead technology and capillary electrophoresis on Applied Biosystems™ DNA sequencers

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Glycosylation plays many important roles in biological processes involving function, pharmakinetics stability, and immunogenicity.As such, it is important to monitor recombinant protein heterogeneity to ensure safety, effectiveness, as well as consistency in manufacturing for biopharmaceuticals. The most widely used methods currently for glycan analysis require time-consuming sample prep, with overnight N-Glycan release steps. Additional purification steps are required to remove excess salts, and there is a requirement for large quantities of protein input. In addition current methods have difficulty producing data on highly sialylated glycans.

We have developed an integrated glycan analysis platform consisting of magnetic bead-based sample prep with multiple dyes for glycan labeling coupled with a multi-capillary CE instrument for parallel analysis of labeled glycans and assay specific software for glycan quantitation. We have eliminated time consuming vacuum centrifugation from the workflow and the use of toxic sodium cyanoborohydride, resulting in a streamlined, automatable method that can process 96 samples in 7-9 hours. The new dyes can resolve glycans that remain unresolved by APTS in CE. The low-maintenance 3500xl CE platform achieves quantitative, high resolution glycan analysis, with high-sensitivity and high-reproducibility.

Here, we report the analysis of highly sialylated glycans usually found on monoclonal antibodies. Furthermore, CE detection capability allows analysis of glycan's as low as femtomoles with APTS, Teal and Turquoise.

(219) Synthesis of rare sugar conjugated glycolipids by combination of chemical reaction and enzymatic reaction

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Rare sugars are monosaccharides that occur small quantities in nature and derivatives of them. They are known to have various effects on biological materials and foods such as inhibiting hyperglycemia, inhibiting cancer cell growth, dental care, and so on. In this study, we aim to synthesis rare sugars conjugated glycolipids.

We synthesised rare sugar conjugated glycolipids by combination of modified Fischer method and reverse reaction of lipase. H_2SO_4-silica (sulfuric acid immobilised on silica) was prepared by mixing diethylether, sulfuric acid and silica gel and evaporation with vacuum condition. We mixed glycerol, sugars (glucose, ribose, allose and rhamnose) and H_2SO_4-silica and heating with stirred. We purified sugar-glycerol conjugate from reactants with silica gel fractionation and concentrated with centrifugal evaporator. Mixture of sugar-glycerol, 2,2,2-trifluoroethyl butyrate and lipase (immobilised on a macroporous acrylic resin) in pyridine were incubated. We purified products by silica gel column chromatography with methanol after the reactants were concentration with centrifuge evaporator. Rare sugar conjugated glycolipids were purified by silica gel column chromatography.

We analysed the products with Fourier transform infrared spectrometer and confirmed characteristic peaks of hydroxyl group and ester at around 3400 cm-1 and 1600 cm-1, respectively. It is indicated that rare sugar conjugated glycolipids were synthesised.

We analysed cytotoxic activity of glycolipids with Cytotoxicity LDH assay kit-WST (Wako). We mixed cell suspension and glycolipids solution in 96 well plate. Then the plate was kept in CO2 incubator at 37°C for 5 h. We estimated rate of plasma membrane damaged cells by measuring lactate dehydrogenase activity. Cytotoxic activity of the glycolipids increased with an increase in the concentration of them.

We measured hemolysis activity of glycolipids. We perpetrated red blood cells (RBC) from sheep blood with centrifugation and washing with PBS. We mixed RBC suspension and glycolipids solution. Mixtures were incubated at 37°C for 5 h with microtube rotator. After centrifugation, we measured absorbance of hemoglobin in supernatant at 540 nm. It is indicated that glycolipids have high affinity to RBC.

(220) Developing Smart Anti-Glycan Reagents Using an Ancient Immune System

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The jawless vertebrates (lamprey and hagfish) have evolved a novel adaptive immune system (AIS), with many similarities to the jawed vertebrate AIS, including the production of antigen-specific circulating antibodies in response to

immunization. However, instead of immunoglobulin (Ig)-based receptors, the jawless vertebrates use leucine-rich repeat (LRR)-based antigen receptors termed variable lymphocyte receptors (VLRs) for immune recognition. VLRs are generated in developing lymphocytes through a gene conversion process, in which random assembly of hundreds of LRR gene segments results in a potential repertoire of $>10^{14}$ distinct antigen receptors. Due to the divergence of lampreys from our jawed vertebrate ancestors >500 million years ago, we hypothesize that lampreys will respond to conserved glycans that are "invisible" to the mammalian AIS due to self-tolerance. To test this hypothesis, we have immunized lampreys with a myriad of mammalian tissues and cell lines and tested the immune stimulated plasma on the Consortium for Functional Glycomics microarray. The results of these experiments suggested that lampreys generate a wide variety of glycan-specific VLRs in response to antigenic stimulation. To isolate these VLRs, we have developed a high-throughput VLR antibody display platform in which VLR antibodies from immunized lampreys are expressed on the surface of yeast cells. VLR expressing yeast are then bound to the glycan microarrays directly, thus enabling us to test the specificity of individual yeast clones against hundreds of glycans simultaneously, and develop an enrichment strategy in which a broad array of glycan-specific VLRs can be identified from the yeast surface display library itself. Subsequently, the bound yeast cells are directly removed from the microarray, the VLR antibody clone is sequenced, and this molecule is expressed as a VLR-IgG-Fc fusion protein that can be used for ELISA, western blotting, flow cytometry, and immunomicroscopy. Thus, by combining yeast surface display with glycan microarray technology, we have developed a rapid, efficient and novel method for generating chimeric VLR-IgG-Fc proteins that recognize a broad array of unique glycan structures with exquisite specificity. *This work is supported by the NIH U01 CA199882.*

(221) Glycopolymers with tunable lectin-binding properties based on self-assembling glycopeptides

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Lectin-carbohydrate interactions participate in numerous biological processes, including fertilization, adhesion and virulence of pathogens, cancer, and inflammation, which has led to increasing interest in these interactions as diagnostic markers and therapeutic targets. Carbohydrate multivalency is a hallmark of high affinity interactions between lectins and natural glycoconjugates, and glycomaterials that can mimic this multivalency are often advantageous for overcoming the characteristic low affinity of monovalent lectin-carbohydrate interactions. However, for glycomaterials to be broadly useful, their chemical and physical composition should be easily modifiable to enable optimization of their lectin-binding properties, namely affinity and specificity. Towards this end, we developed glycopolymers with easily modifiable carbohydrate topology and chemistry based on glycopeptides that self-assemble into nanofibers. Specifically, we synthesized a family of glycopeptides, referred to as GlcNAc-Q11," which consists of (GlcNAc)-Asn conjugated to a peptide that self-assembles into beta-sheet rich nanofibers (QQKFQFQFEQQ, "Q11") via a serine-glycine linker. Glycopolymers with different carbohydrate lateral densities are prepared via co-assembly of GlcNAc-Q11 and Q11 at various molar ratios, while radial distance of the carbohydrate and polymer is varied by increasing the serine-glycine linker length from 1 to 4 repeats. Carbohydrate chemistry is varied by enzymatically treating GlcNAc-Q11 nanofibers with beta-1,4-galactosyltransferase in the presence of a sugar donor, uridine diphosphate galactose (UDP-gal), to yield N-acetyllactosamine (LacNAc-Q11). We systematically varied chemical and physical features of glycopeptide nanofibers to identify correlations between glycopolymer composition and lectin-binding properties. GlcNAc-Q11 glycopolymers bound to wheat germ agglutinin (WGA) and griffonia simplicifolia lectin II (GS II) with significantly different affinities, which depended on carbohydrate lateral density but not on radial distance from the nanofiber. In particular, increasing carbohydrate density decreased WGA binding affinity and completely abolished GS II binding, likely due to steric hindrance. Finally, LacNAc-Q11 glycopolymers failed to bind WGA, but bound galectin-1 with high affinity in a carbohydrate density-dependent manner. Thus, self-assembling glycopeptides provide glycopolymers with readily modifiable carbohydrate content that can be tailored to finely tune their lectin-binding properties. We envision that these glycopolymers will provide the basis for biomaterials that can harness specific lectin-carbohydrate interactions for various therapeutic applications.

(222) Self-assembled lectin-binding glycopolymers for immunomodulation

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Recognition of cell surface glycans by lectins modulates numerous aspects of innate and adaptive immune cell function, which has led to increasing interest in lectins as targets for immunotherapy and immunomodulation. However, lectin-glycan interactions are a double-edged sword in immunology - they can provide outside-in signals that direct normal, healthy immune responses, such as resolution of inflammation and antigen-specific tolerance, yet can be hijacked to promote progression of immunopathologies, such as tumor immune privilege and viral infection. Thus, effective therapeutics must be designed to selectively enhance or inhibit specific interactions between lectins and cell-surface glycans according to the intended immunological outcome. Toward that end, we create glycopolymers that can non-covalently bind to lectins in a carbohydrate-dependent manner from synthetic glycopeptides that self-assemble into β-sheet

nanofibers in water. The highly multivalent presentation of carbohydrates along the nanofiber enhances lectin-carbohydrate binding affinity, which can be tuned via co-assembly of glycopeptides and non-glycosylated peptides at different molar ratios. In addition, varying carbohydrate chemistry via glycosyltransferase enzymes provides control of nanofiber lectin-binding specificity. In biological media, glycopeptide nanofibers can capture lectins and inhibit their activity as extracellular signals. For example, nanofibers modified with N-acetylglucosamine (GlcNAc) can inhibit lectin-mediated T cell apoptosis via selective, high-affinity binding to wheat germ agglutinin (WGA), while nanofibers modified with N-acetylactosamine (LacNAc) can inhibit apoptosis induced by galectin-1. Glycopeptide nanofibers can also be fabricated into micron-sized particles (i.e. "microparticles") loaded with lectins that are controllably released via reversible lectin-nanofiber binding. For example, WGA was efficiently encapsulated into microparticles fabricated from GlcNAc-modified nanofibers, and was released from the microparticles into bulk aqueous media at tunable rates that were governed by the concentration of GlcNAc within the microparticle. WGA released from microparticles induced T cell apoptosis with comparable activity to stock lectin, suggesting that glycopeptide microparticles may be useful as vehicles for local delivery of therapeutic lectin payloads. We envision that self-assembled glycopeptide nanofibers having lectin-binding properties that can be finely tuned will be broadly useful for creating therapeutics that can enhance or inhibit particular lectin-glycan interactions to elicit desired immune responses.

(223) Oligosaccharide Microarrays with Neoglycolipid Probes Prepared from Synthetic Amino-Terminating and Naturally-Derived Amino Acid-Terminating Oligosaccharides

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Oligosaccharide microarrays are playing important roles in detection of protein-carbohydrate interactions in health and in disease processes including infection, inflammatory reactions, immune responses and cancer. The neoglycolipid (NGL)-based microarray system developed in the Glycosciences Laboratory at Imperial College is one of the two major platforms that are available to the international community for screening analysis of carbohydrate recognition systems, the other being the platform developed by the Consortium for Functional Glycomics (CFG). The NGL platform uses a unique non-covalent immobilization of the glycan NGL probes (derived predominantly from natural sources) in a liposomal formulation that enables an element of mobility simulating glycoconjugate presentation at the cell surface. In the CFG platform immobilization of glycans (predominantly synthetic) is by covalent means. The two platforms have served well the biological and biomedical communities.

NGLs are prepared from reducing sugars by conjugation via their (hemiacetal) aldehyde groups with amino-terminating lipid reagents. These reducing sugars are typically from glycans released from glycoproteins, glycolipids or polysaccharides. The CFG arrays are prepared by direct covalent immobilization of the amino-terminating sugar onto the microarray slide through a functionalized surface.

We have now designed a new aldehyde-terminating lipid reagent in order to conjugate to amino-terminating glycans. In this communication, we describe the synthesis of the benzaldehyde-terminating lipid reagent, which is highly active towards the amino functionality. We demonstrate its usage in preparation of NGL probes from a wide range of amino-terminating glycans: synthetic amino-terminating (including alkyl and aromatic amines) and aminooxy-functionalized glycans, and naturally derived glyco-amino acids and glycopeptides. To validate the approach we have evaluated bindings to the new types of NGLs with a wide-range of carbohydrate-recognition systems, including plant lectins, monoclonal antibodies, microbial carbohydrate-binding modules, proteins of the immune system and adhesion proteins of viruses.

The new strategy will enable expansion of the repertoire of the NGL library considerably (from the current >800 probes) in number and structural diversity, leading to wider applications. This will also provide basis for a direct comparison of glycan array platforms using the same amino-terminating glycans for covalent and for non-covalent immobilization.

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(224) Predicting N-glycan processing based on enzyme-glycan accessibility

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Predicting N-glycan processing 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 two systems (a protein disulphide isomerase named Pdi1 [1] and a hemagglutinin (HA) from influenza A [2]). In the case of Pdi1p, 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.

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(225) Glycomimetic Approach to Structural Modification of Lysine Residues in Therapeutic Peptides

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The growing demand for biopharmaceutical drugs is attributed to the discovery of new recombinant production methods, which have reduced drug production costs. Therapeutic proteins and peptides are biopharmaceuticals that exhibit high specificity compared to other drugs. They are effective in correcting disorders and may be used as vaccines, enzymes, or regulatory factors; however, they are susceptible to enzymatic degradation, immunogenicity and rapid elimination. In order to overcome these challenges, the protein or peptide structure is modified through conjugation to polymers or coupling to small organic molecules.

In proteins or peptides, the Nα amino group of lysine is engaged in the polymer backbone except for the N-terminal lysine residue; therefore Nα-p-tosyl-L-lysine methyl ester hydrochloride was chosen to mimic the lysine residue in a polypeptide. The tosyl-L-lysine was coupled with a glucose-derived aldehyde by reductive alkylation. Hydrolysis of the product liberated an aldehyde that underwent an intramolecular reductive alkylation to cap the Nε amine with a 2,4-hydroxypiperidine ring. Having established the suitable conditions for this modification, similar conditions were applied to a therapeutic tripeptide, KPV, derived from the C-terminal sequence of the α-melanocyte stimulating hormone (α-MSH). Both KPV and α-MSH are C-terminal amidated and N-terminal acetylated. They are reported as having anti-inflammatory and antimicrobial activities. Three compounds were synthesized: Ac-KPV-NH_2, to be used as a control in determining the effects of the modification, Glyco-KPV-NH_2, and K(Glyco)PV-NH_2.

To check the effect of our modification on the antimicrobial activity of the tripeptide, Staphylococcus aureus cells were separately exposed to Ac-KPV-NH_2, Glyco-KPV-NH_2, and K(Glyco)PV-NH2. The biological tests did not show activity for these compounds, including Ac-KPV-NH_2, which has been reported to have activity. There have been conflicting results published in the literature concerning the antimicrobial activity of this compound. Since we could not ascertain the effect of our modification using the antimicrobial activity tests, we are currently investigating the same using anti-inflammatory assays. We anticipate that the activity will be maintained or improved due to the amphiphilic nature of the modifier, which enhances the pharmacodynamic properties of the drug.

(226) Using a Modification Site Database To Improve Glycopeptide Identification

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A spectrum of different glycans decorate each glycosylation site in a protein. As a result, if a given glycopeptide is identified, there is a high probability that other glycoforms of the same modification site are also present in the sample. Also, there are many glycosylation sites identified in the literature. If information about known modification sites could be used to direct glycopeptide data analysis tools, then this should lead to increased glycopeptide identification. In this study we adapted Protein Prospector to be able to use a modification site database to filter sites considered as modified during searching. Different strategies for populating the modification site database were also compared.

Known glycosylation sites were extracted from the .dat file associated with UniProtKB and used to populate a modification database. Alternatively, glycosylation sites identified by in-house glycopeptide analysis studies were extracted from Protein Prospector results. The effects of applying modification site knowledge to filter modified peptides considered as glycosylated were evaluated by comparing to other searching strategies, such as restricting the proteins considered.

Filtering of considered modification sites at the protein, peptide and site level all lead to an increased number of glycopeptide spectra identifications, in some cases more than doubling the number of glycopeptides identified, while still maintaining high reliability identifications. Filtering at the site-level lead to the largest number of glycopeptide identifications, but can introduce a higher false localization rate of modification site assignments, particularly for O-linked glycosylations. Restricting to the modified peptide level is able to address this weakness, so seems the best compromise for increasing the number of identifications while maintaining reliability in reported results. Discussion will be provided of whether the use of a site localization database will be equally beneficial for all search engines.

The power of this approach will only be fully realized upon the construction of comprehensive site databases. As such, populating of public databases with modification site information, particularly O-glycosylation data where sites cannot be predicted, will be an important step to advance the field.

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(227) Recognition Tunneling Nanopores for Sequencing of Glycosaminoglycans

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Glycosaminoglycans (GAGs) are long linear polysaccharides found at the surface of cells and in the extracellular matrix. Though glycosaminoglycans (GAGs) have been known as a key player in biological processes such as cell signaling and development, blood coagulation, tumor growth, and microbial infection, the structural analysis of GAGs remains one of the most challenging research area in glycoscience because of their size and semi-random nature of sulfation modification. There is an urgent need for a technique that provides information on sequences of GAGs. Our goal is to develop a method to analyze structure of GAGs using recognition tunneling nanopores.

Nanopore is an orifice with a nanometer diameter and functions as nanofluidic channel for the flow of ions and the transport of biomolecules. Through decades of research efforts, the nanopore has become a practical tool for sequencing of nucleic acids. We have developed a recognition tunneling (RT) technology for sequencing DNA with a single base resolution when incorporated into a solid-state nanopore. Recently, we have demonstrated that RT can distinguish among mono- and di-saccharides. Based on these findings, we have developed a scheme for sequencing GAGs by RT embedded in nanopores. Herein, we report GAGs translocation through solid-state nanopores and their translocation speed control. We have translocated a series of heparin and chondroitin sulfate polysaccharides through nanopores with a diameter of ~3.0 nm on 30 nm thick silicon nitride membrane and analyzed the translocation duration, blockade current amplitude, and event signal shape. We observed that the length of GAG is crucial to observation of the translocation signals, and translocation dwell times as well as blockade currents of GAGs are correlated to GAG sizes as well as sulfation densities, making these parameters potential markers for analyzing GAG samples. We also demonstrates that the translocation speed can be controlled by pore modification. In the next step, we will use a RT nanopore device for reading sequences of GAGs.

(228) Development of a 5-Minute Deglycosylation Method and Instant Labeling Dye for High-throughput N-Glycan Analysis by Mass Spectrometry

Aled Jones, Michael Kimzey, John Yan, Vaishali Sharma, Andres Guerrero, Alexander Gyenes, Justin Hyche, Emily Dale, Ted Haxo, Sergey Vlasenko
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The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics. This makes the characterization of N-glycans an essential part of the biotherapeutic development process. Analysis of N-glycans typically involve the labeling of enzymatically-released glycans with a tag to allow for fluorescence detection; a process that often requires numerous hours or days to complete. In addition to fluorescence (FLR) detection, mass spectrometry (MS) is also often utilized. Unfortunately, many of the commonly-used fluorescent tags are limited with regard to MS sensitivity.

We present a rapid N-glycan sample preparation workflow which uses a 5-minute in-solution digestion, instant labeling, and cleanup of excess label and denaturant prior to analysis. The workflow can be completed in as little as 45 minutes and includes InstantPC, a new instant glycan labeling reagent that provides markedly increased MS and FLR sensitivity. InstantPC-labeled N-glycans are suitable for hydrophilic interaction liquid chromatography (HILIC) utilizing both FLR and MS detection allowing flexibility for screening applications as well as in-depth characterization of N-glycans.

(229) An Integrated System for High-throughput, User-friendly N-Glycan Analysis Using Rapid Separation by Capillary Electrophoresis

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Glycan characterization is becoming necessary in the earliest stages of biotherapeutic cell line development, to the point where cell culture screening often requires glycan profiling. This entails significantly increased throughput for sample preparation, analytical instrumentation, data processing and expertise in glycan characterization. Unfortunately, these factors can cause a bottleneck to results.

Here we present a glycan analysis solution that provides rapid sample preparation and analysis combined with a simplified data processing approach. The sample preparation includes a 5-minute deglycosylation step to release N-glycans, followed by glycan labeling and cleanup, and may be completed in under 1 hour. Labeled N-glycans are separated using a small and user-friendly capillary electrophoresis (CE) instrument, with a run time of 2 minutes per sample. Using custom software, glycan migration times are processed into glucose unit (GU values), and compared against a library of known N-glycan GU values. The relative percent area for each peak is automatically calculated. This process enables relative N-glycan quantification for up to 96 cell culture samples within a single workday.

(230) High-Throughput Milk Oligosaccharide Analysis Using a Rapid Cartridge-Based Capillary Electrophoresis Instrument

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Milk oligosaccharides (OS) have received much interest for their biological roles as prebiotics, antimicrobial agents and

modulators of the immune system. Milk OS are structurally complex molecules that exhibit multiple isomeric forms, and are present with a large dynamic range of concentration in milk and dairy products. Many different analytical approaches and techniques have been used to characterize and quantify milk OS, each exhibiting different advantages and disadvantages.

In this study we employed Gly-Q, a simple, robust and low-maintenance analytical platform developed for the high-throughput analysis of carbohydrates. Sample preparation starting from milk or other dairy products is rapid and automatable, allowing the processing of up to 192 samples in one day. Once prepared, labeled oligosaccharide samples are analyzed using the Gly-Q instrument in a high-throughput and rapid way: each run takes less than two minutes. In this study, human and bovine milk, as well as developmental dairy products enriched in milk OS, were prepared and analyzed using Gly-Q. The results were compared with milk OS data obtained with more conventional (and more time-intensive) analytical techniques such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) or liquid chromatography coupled to mass spectrometry (LC-MS). In our tests the analytical speed of the Gly-Q instrument did not compromise milk OS compound resolution or sensitivity compared to the other analytical techniques tested.

Milk OS data obtained with the Gly-Q system was reproducible, sensitive, and compared favorably to existing analytical techniques. Because of the rapidity of measurement and inherent high throughput, this new platform will prove to be an invaluable tool in the fields of nutritional and medical sciences, as well as in the dairy industry R&D where it could be used to assist with new product development.

(231) Simultaneous glycosyl composition analysis of polysaccharides of varying stability and solubility by derivatization with methyl groups

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Glycosyl composition analysis is an essential part of the elucidation of polysaccharide structure. The current methods are reasonably reliable but suffer from a number of shortcomings, including degradation of released monosaccharides during acid hydrolysis and lack of detection of insoluble polysaccharides. The purpose of our research is to develop a single method of composition analysis which will work for all polysaccharides, including those that are insoluble. Currently, proper hydrolysis of different groups of polysaccharides requires variable hydrolysis conditions. For example, hydrolysis of crystalline cellulose requires harsher conditions and longer reaction times than hydrolysis of pectin, so that an accurate, simultaneous determination of the components of these two polysaccharides is not possible. Our research addresses these problems by first performing a permethylation of the polysaccharides, which increases their solubility in the following hydrolysis step, while at the same time stabilizing released monosaccharides towards acidic degradation, leading to more accurate composition results. An additional benefit of the present method is that a portion of the permethylated polysaccharide can be taken on to linkage analysis, so that both composition and linkage analysis come from the same sample, allowing a more direct comparison. The proposed method meets the widespread need for a more accurate determination of cell wall and other polysaccharides.

(232) An evolutionary systems approach to investigate sequence-structure-function relationships in Glycosyltransferases

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Glycosyltransferases (GTs) are a broad class of proteins involved in the transfer of a glycosyl group from a donor molecule to an acceptor. As such, they are involved in a wide range of biological functions through their roles in glycosylation modifications, synthesis of cellular receptors, biosynthesis of polysaccharides, glycolipids and glycoproteins. There are 3 main structural classes (GT-A, GT-B and GT-C) and about 100 different functional classes of GTs (as per the CAZy database, accessed 24 August 2016), each one specialized in their function with their own characteristic features. Previous studies have provided a basis for a broad classification of GTs. However, a residue level understanding of how individual GT families evolutionarily diverged is currently missing. Here, we use a Bayesian statistical approach to classify GT-A fold sequences into functional categories based on the patterns of conservation and variation in large multiple sequence alignments. We use patterns unique to each GT family within the GT-A fold as a conceptual starting point for investigating their sequence-structure-function relationships. Implementing these methods, we can further pinpoint contrasting, similar and co-evolved features that differentiate, associate and functionally relate the GT-A fold families respectively. Here we present a preliminary analysis that highlights the presence of co-conserved features that distinguish multiple GT-A fold families. Structural analysis of co-conserved features suggest novel models of regulation and substrate specificity determining residues in major GT groups and families. These observations help understand how these key differences impact the biological activity thus contributing to the evolution of family specific divergence and functional specificity of GTs. Further, we plan to incorporate metabolomic and glycomic data from mutants of these GT family sequences to relate these unique patterns to functional unit changes. This will add an important layer of information that can help us elucidate the functional roles of the features and the protein families as a whole.

(233) GLYCAM16: A major update to the GLYCAM biomolecular force field

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Abstract: The GLYCAM force field family, especially GLYCAM06, has been extensively employed by the scientific community in simulations of biomolecules. GLYCAM06 successfully predicted the kinetic and thermodynamic properties of carbohydrates, as well as other biomolecules. However, recent reports demonstrate a weakness in the ability of GLYCAM06 to accurately reproduce the conformational properties of some carbohydrate rings, such as methyl β-D-xylopyranoside. Here, we present GLYCAM16, an update to GLYCAM06 that addresses these issues. A new atom type "Op" for ring oxygen atoms in pyranoses has been introduced in GLYCAM16, to differentiate ring oxygen atoms from those in glycosidic linkages. Parameters for ring-related bond angles have been derived from mono-pyranosides. With these updates, GLYCAM16 could correctly reproduce the average ring angle values from crystallographic structures in molecular mechanics (MM) energy minimized structures, and the conformational properties of pyranose rings measured from NMR in molecular dynamics (MD) simulations.

(234) High-throughput characterization of N-linked glycosyltransferase peptide and sugar specificities enabled by cell-free protein synthesis and SAMDI mass spectrometry

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In recent decades, advances in high-throughput analysis technologies such as Next-Generation Sequencing, RNA-seq, and quantitative proteomics have far outpaced enzymatic characterization assays. This gap is especially evident in the field of glycobiology where the unique challenges of glycan analysis and the heterogeneity resulting from complex glycosylation pathways *in vivo* often make methods for biochemical characterization of glycosyltransferases (GTs) costly and tedious. Indeed, less than 1% of putative GTs in the Carbohydrate-Active enZYme (CAZY) database have been biochemically characterized. To address this limitation, we have developed an *in vitro* platform for the high-throughput expression and characterization of GTs using *E. coli* based Cell-Free Protein Synthesis (CFPS) and Self-Assembled Monolayers for Desorption Ionization (SAMDI) mass spectrometry. A key advantage is that the open reaction environment of the cell-free system allows for rapid optimization and manipulation of protein synthesis and glycosylation reaction environments and permits direct application to SAMDI-MS which is a label-free, high-throughput experimentation tool. Furthermore, *E. coli* lysates lack native protein glycosylation machinery, providing a blank canvas for detailed characterization. Here we report the *in vitro* expression and characterization of the sugar and peptide specificities of five cytoplasmic N-linked glycosyltransferase (NGT) homologs from pathogenic bacteria, including two without previous functional characterization, at unprecedented throughput and depth. Our CFPS system achieved soluble titers of 130-1080 μg/mL of all five NGT homologs and allowed for accurate protein quantification and characterization without purification. By applying these cell-free reactions directly to functionalized SAMDI monolayers, we analyzed over 2,600 unique peptide acceptor substrates, collecting mass spectrometry data from over 4,200 unique experimental conditions. Despite sequence identity ranging from 40% to 77% compared to the most thoroughly characterized NGT homolog, *Actinobacillus pleuropneumoniae* NGT (ApNGT), sugar and peptide substrate preferences were well conserved. Looking forward, we envision that our framework will meaningfully contribute to new understanding of diverse glycosyltransferase families and help dissect their roles in important biological processes.

(235) Evolutionary analysis for O-GlcNAcylated proteins by clustering method

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O-GlcNAcylation, the attachment of a single N-acetylglucosamine to serine/threonine residues of proteins by O-GlcNAc transferase (OGT), is essential for protein function in eukaryotes. OGT was shown to be essential for the viability of embryonic stem cells. In spite of these important roles, no consensus sequence for O-GlcNAcylation had been found so far. In addition, it was found that those O-GlcNAcylated residues are not conserved like non-O-GlcNAcylated residues. However, owing to their crucial roles in protein function, O-GlcNAcylated residues are expected to be at least partially evolutionarily conserved. There are many parallels between phosphorylation and O-GlcNAcylation; attachment occurs to serine/threonine residues, half-life is short, occurrence is infrequent in structured regions, whereas dominant in intrinsically disordered regions (IDRs).

In this study, we clustered O-GlcNAcylated proteins to investigate the relation between their evolutionary conservation and function for proteins. We used the experimental data of human O-GlcNAcylated proteins. Firstly, KEGG ortholog cluster was used for homology search in eukaryotes with genomes completely sequenced. Secondly, O-GlcNAcylated residues in orthologous proteins were checked for conservation using multiple sequence alignment. Finally, O-GlcNAcylated proteins were clustered by the degree of their conservation.

In the clustering result, O-GlcNAcylated proteins were clustered into 9 groups. Both ratios of O-GlcNAcylation within IDRs and co-occurrence of O-GlcNAcylation/phosphorylation at identical residues were high in evolutionarily young clusters. Comparison of the degree of conservation in

OGT and O-GlcNAcase (OGA), the degrading enzyme of O-GlcNAc, showed that OGT was more widely conserved than OGA. In addition, approximately half of O-GlcNAcylated proteins were as conserved as OGA. These results suggest that a lot of O-GlcNAcylated proteins were acquired along with OGA. We are currently analyzing the relation between evolution and function for O-GlcNAcylated protein in each cluster.

(236) Comparing Detector Response for 2-Aminobenzamide Labeled N-Glycans

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One common method for separating the asparagine-linked oligosaccharides (N-glycans) released from glycoproteins is to attach a fluorophore by reductive amination and then separate the labeled N-glycans by either reversed-phase or hydrophilic interaction chromatography and then detect them by their fluorescence. 2-aminobenzamide (2-AB) is perhaps the most common fluorophore used for this purpose. Another common method of N-glycan analysis is the separate the native oligosaccharides by high-performance anion-exchange chromatography and detect them by pulsed amperometry (HPAE-PAD). Some analysts have combined the techniques. They first label the N-glycans with 2-AB and then separate the oligosaccharides using the columns and mobile phases commonly used for HPAE-PAD, but detect by fluorescence (HPAE-FLD). One published version of such a method is in United States Pharmacopeia (USP) proposed General Chapter <212>. The scientific literature shows that 2-AB labeled N-glycans can also be detected by PAD. Therefore, during our testing of the reference standards for <212> with the proposed chromatography method in <212> we took the opportunity to install an electrochemical detector after the fluorescence detector. In this manner we could directly compare the responses of 2-AB labeled N-glycans from bovine fetuin and alpha-1-acid glycoprotein. Looking at the largest peaks, the fluorescence response was approximately 70X greater than the PAD response. We were also able to make a less direct comparison of the PAD response of unlabeled N-glycans from the same two glycoproteins. This comparison showed that the PAD response is still less than, but closer to, the fluorescence response, suggesting that the addition of the fluorophore reduces an oligosaccharide's PAD response. This poster will also look at the impact of certain oligosaccharide structural features on the response of the two detectors. These data will aid scientists comparing results from N-glycan analysis techniques.

(237) SweetNET: A bioinformatics workflow for glycopeptide MS/MS spectral analysis

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Glycoproteomics has rapidly become an independent analytical platform bridging the fields of glycomics and proteomics to address site specific protein glycosylation and its impact in biology. Current glycopeptide characterization relies on time-consuming manual interpretations and demands high levels of personal expertise. Efficient data interpretation constitutes one of the major challenges to be overcome before true high-throughput glycopeptide analysis can be achieved. The development of new glyco-related bioinformatics tools is thus of crucial importance to fulfil this goal.

Here we present SweetNET: a data-oriented bioinformatics workflow for efficient analysis of hundreds of thousands of glycopeptide MS/MS-spectra. We have analyzed MS data sets from two separate glycopeptide enrichment protocols targeting sialylated glycopeptides and chondroitin sulfate linkage region glycopeptides, respectively. Molecular networking was performed to organize the glycopeptide MS/MS data based on spectral similarities. The combination of spectral clustering, oxonium ion intensity profiles and precursor ion m/z shift distributions provided typical signatures for the initial assignment of different N-, O- and CS-glycopeptide classes and their respective glycoforms. These signatures were further used to guide database searches leading to the identification and validation of a large number of glycopeptide variants including novel deoxyhexose (fucose) modifications in the linkage region of chondroitin sulfate proteoglycans.

(238) Structural Characterization of a High Molecular Weight Sulfated Galactan Obtained from the Tunic of the Ascidian Microcosmus exasperatus

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Several studies have shown that marine invertebrates produce unique sulfated polysaccharides, which can be exclusive according to each organism. In the last decades,

different sulfated polysaccharides have been isolated from the body and the tunic of ascidians, a group of tunicates that have shown to be an important source of bioactive natural products with antitumor, antimicrobial or anti-inflammatory effects. Among these polysaccharides, high molecular weight L-galactans are the main constituents of the tunic in different ascidians. These glycans can present distinct structures due to variation in charge density and chemical composition. For instance, the α-L-galactan from *Herdmania monus* is 3-sulfated and 4-linked whereas the L-galactan from *Ciona intestinalis* is poorly sulfated. This study shows the first report of the extraction and chemical characterization of a sulfated galactan from the tunic of *Microcosmus exasperatus*. This polysaccharide was extracted and purified through proteolytic digestion, ethanol precipitation, dialysis and freeze-thaw cycles. The average molecular weight was determined by high-performance size exclusion chromatography (HPSEC) analyses. In addition, the structure of this compound was identified through 1D and 2D NMR spectroscopy and methylation analysis. Our results showed the presence of a high molecular weight sulfated polysaccharide (435 KDa) that is mainly composed of (1→4)-linked α-Galp units, which can be C-3/C-6 substituted by sulfate groups or by galactose units. The identification of this kind of polymer in the ascidian *M. exaspteratus* ratifies that sulfate and galactose are incorporated into the sulfated polysaccharides of the tunic, as it is the case of the typical galactans previously isolated. Even though the structure of galactans from ascidians has been identified, their biological application remains poorly explored. The structural characterization is a crucial step for further biological analysis; it opensup a new research field towards the discovery of marine polysaccharides with potential pharmacological activity.

(239) Elucidation of the mechanism of capsular polysaccharide recognition by Campylobacter jejuni bacteriophages

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Bacteriophages represent the most abundant forms of life on Earth. With an estimated 10^{31} virion particles worldwide, phages are believed to have hosts in every bacterial genus. Through their receptor binding proteins (RBPs), phages recognize and bind specifically to their host species. This high degree of specificity presents an opportunity for using phages or phage derivatives as antimicrobials. Phages have a propensity to bind complex carbohydrates, since these are commonly displayed on bacterial surfaces, making RBPs an excellent source of novel glycan binding proteins.

Campylobacter jejuni is a prevalent foodborne pathogen with an extensive array of glycoconjugates presented on the outside of the cell. Among these glycans are capsular polysaccharides (CPS), which are bound by *C. jejuni* phages prior to infection. We tested phage infectivity of several *C. jejuni* mutants with defects in glycoconjugate production, including flagellar O-glycans, lipooligosaccharides, N-linked glycoproteins, and CPS. *Campylobacter* phages NCTC 12673 and NCTC 12674 were unable to infect the CPS mutant *kpsM*. By testing several mutants with deletions of peripheral modifications, we determined the phages recognize the CPS backbone. However, comparison of the CPS structures among those serotypes susceptible to phage lysis did not identify even a single common monosaccharide. Therefore, we hypothesized that the phage RBPs recognize a conformational epitope on the CPS structure.

To further understand the mechanism of this interaction, we sought to identify and characterize the CPS-binding phage RBP from the NCTC 12673 genome. Since there is no sequence homology in thephage genome compared to other described phage RBPs, we identified CPS binding candidates based on Phyre 2-predicted structural homology to glycan binding proteins. After expression and purification of these proteins in *E. coli*, we identified one novel *C. jejuni*-binding protein that is also capable of phase-variation. The characterization of this phage protein will be described.

(240) Dissecting glycan diversity across animal species by mass spectrometry

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The modern revolution in molecular biology is being driven to a large extent by advances in methods for analysis and manipulation of DNA, RNA, and proteins, biomolecules whose syntheses are all driven through the copying and translation of templates. Glycosylation is one of the most common post-translational modifications of proteins and is not driven through a guiding template, but rather through the concerted action of a series of biosynthetic enzymes whose expression varies among different cell types and developmental stages in all animals. Complex carbohydrates, also known as "glycans," serve key functions as receptors and modulators of cell-cell and cell-pathogen interactions as well as regulators of diverse receptor-mediated cell signaling events. Unlike genomics and proteomics, which have benefitted from rapid technological advances over the last 30 years, the analysis of a complete glycome, defined as the entire catalogue of glycoprotein and glycolipid glycans

present in a biological sample, remains technically challenging. Recent advances in carbohydrate chemistry, chemical biology, and mass spectrometric (MS) techniques have opened the door to rapid progress in uncovering the function and diversity of glycan structures associated with human health and diseases. These strategies can be equally well applied to advance veterinary health care research. Interestingly, the basic biosynthetic pathway that initiates the production of a glycoprotein is conserved across a wide evolutionary range of living organisms. Subsequent trimming, extension, and post-synthetic modifications of this common core are highly variable across species and across cell types within any one species. While the conservation of core glycosylation indicates the essential nature of this protein modification, the diversity of glycan structural elaboration across species suggests that glycosylation patterns may contribute to evolutionary diversification of animals. Therefore, defining glycan diversity across animal species will lead to understanding the function of glycans at every level of phylogeny, between species, within populations of the same species, and also among different molecules and cell types within the same organism. We profiled the structures of complex glycans in a broader variety of animals to understand "How animal species developed such a heterogeneous structural glycan diversity in evolution," using comprehensive and highly sensitive MS approaches.

(241) Performance Evaluation of Orbitrap Fusion Lumos And Orbitrap Fusion For Glycopeptide Analysis

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Large scale intact glycopeptide analysis remains challenging due to complexities associated with the glycopeptide structure. The challenge is further compounded by the fact that traditional fragmentations are not ideal for glycopeptide sequencing. The emergence of ETD, and by extension EThcD have alleviated a lot of these issues. Here we present a performance evaluation comparison of next generation Orbitrap mass spectrometers for glycopeptide analysis. Parameters and workflows will be presented that highlight large scale glycoproteomics.

Glycopeptides were enriched from human serum and HeLa lysates and analyzed on an Orbitrap Fusion and Orbitrap Fusion Lumos mass spectrometer. Various ETD reaction times, AGC target values, isolation windows, supplemental activation collision energy and RF were tested to maximize identification.

Recently, a novel fragmentation called EThcD has been introduced that appears to be advantageous for glycopeptides – increasing sequence coverage and glycosylation site localization. Our initial experiments focused on optimizing ETD parameters to improve glycopeptides data on Orbitrap Fusion MS. Typically, longer ETD reaction times were needed for glycopeptides relative to conventional peptides. Various ETD reaction times, fixed or varied, dependent upon charge states were tested to maximize spectral quality. Similarly, we also optimized EThcD parameters, as glycopeptide fragmentation can be maximized by varying supplemental activation collision energy. The primary focus of our experiments was on ETD and EThcD, however, we observed that the quality of HCD spectra were superior to spectra acquired on other platforms for intact glycopeptides. Typically, b and y ions generated from peptide backbone of a glycopeptides are low abundant and are difficult to detect on mass spectrometers. But in these instruments we could easily detect and use them for sequencing.

Our next set of experiments involved optimizing Orbitrap Fusion Lumos parameters. In total 11 parameters were tested with 21 individual runs to maximize performance. After optimization of parameters, experiments were conducted on both platforms to examine performance relative to each other. Overall, Lumos identified 9% more unique glycopeptides relative Orbitrap Fusion by ETD, 43% more by EThcD and 49% by EThcD over ETD. The increase in identification came from large glycopeptides which are challenging in mass spectrometry.

Glycosylation, specifically changes in glycosylation, is a hallmark of cancer. Unfortunately, proteomics studies tend to ignore this particular PTM. Even in unenriched samples, 15-20% of spectra are glycopeptides. Using HeLa as model we explored the possibility of sequencing these modifications in discovery proteomics experiments.

(242) Quantum mechanical studies of glycans using fragment molecular orbital method

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Evaluating highly reliable structure and energies are essential to understand the function of the proteins. Theory plays a major role in this area of science, and especially studies on protein-ligand interactions made remarkable achievements. In recent years, we have been performing high-level quantum mechanical (QM) studies using the Fragment Molecular Orbital (FMO) method to analyze number of protein-ligand complexes.[1] FMO-QM method is a fragmentation method through which a large biological system, such as protein, is divided into fragments, for example, each amino acid as a fragment, and the total properties of the large systems are derived in a many-body expansion by combining the properties of fragments.[2,3] FMO becomes one of the successful

theoretical methods to reliably study the large biosystems such as proteins. In the present investigation, we adopted the FMO-QM methodology to study theoretically sugar chains in glycoproteins at the correlated RI-MP2/cc-pVDZ level. Computational approach of interaction analysis between sugar chains and lectin become crucial because these sugar chain moieties control and relate with many biological functions and molecular recognition. For example, human and avian influenza virus specially recognize corresponding sialic acid receptor, α2,6-linked Neu5Ac-Gal and α2,6-linked Neu5Ac-Gal, respectively. Here we performed total electronic calculation based on the FMO method for the glycans. Normally, FMO scheme divides the protein into small fragments at the C_α atom of each amino acid residue and such fragmentation has no unphysical caps in the theoretical point of view and hence FMO calculation produces reliable energetic results. However, such a simple fragmentation in glycan system has not been established because glycosidic bonds in glycan are various kinds of types. Implementation of a new fragmentation method using PyMOL plugin program is shown to divide automatically sugar chains to fragments and applied to actual FMO calculation for glycans.

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(243) Complete Protein Deglycosylation Using a New Mass Spectrometry-Compatible Protein Deglycosylation Mix

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Detecting glycoproteins and discerning their degrees of glycosylation is critical to elucidate their roles and mechanisms in binding, recognition, signal transduction, and transport. There are analytical challenges associated with these studies, including (1) *N*- and *O*-glycans add a tremendous degree of heterogeneity to the proteins and thus affect fundamental protein attributes, (2) protein ID and proteomics experiments are often compounded by prevalent glycosylation, causing low coverage, less confidence, and limitations in quantitation.

Mass spectrometry of *N*- and *O*-glycoproteins is simplified after deglycosylation. However, chemical methods (such as beta-elimination) are not suitable since they can degrade the polypeptide chain. Enzymatic reagents that completely remove *N*-and *O*-glycans under mild, MS compatible conditions allow the recovery of a clean deglycosylated protein for downstream analysis.

Here we report the use of a protein deglycosylation enzyme cocktail that, in parallel with PNGase F, readily showed whether *N*- or *O*-glycans were present in a variety of proteins. Samples were analyzed by SDS-PAGE and/or MS to determine the loss of glycan modification. This optimized combination of five recombinant enzymes was effective for 14 proteins tested. This mix can be further expanded in combination with additional exoglycosidases to remove unusual *O*-glycan epitopes. We also demonstrate that the treatment of a complex system such as human serum by this protein deglycosylation mix generates more protein hits and better peptide coverage.

(244) In-depth site-specific N- and O-Glycosylation analysis of human C1-Inhibitor reveals extensive mucin-type O-glycosylation

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Human C1 inhibitor is a serine protease inhibitor and the major regulator of the contact activation pathway, as well as the classical and lectin complement pathways. In fact, it is one of the most heavily glycosylated plasma glycoproteins, but only very little is known about the glycosylation features and their biological role.

We performed the first detailed site-specific N- and O-glycosylation analysis of C1-Inh using a panel of glycomics and glycoproteomics workflows. The protein was digested with a variety of proteases and glycopeptide samples were partly treated with PNGase F and exoglycosidases. Glycopeptide analysis was performed using C18-[porous graphitized carbon (PGC)]-LC-ESI-QTOF-MS/MS and C18-LC-ESI-ion trap-MS/MS under different fragmentation regimes such as stepping-energy collision-induced dissociation (CID) and electron-transfer dissociation (ETD).

In total, 27 O-glycosylation sites featuring core 1 O-glycans were identified on C1-Inhibitor. While ten of them could be located with their exact position, one glycosylation site was found to be either Thr27 or Ser28, next to a heavily O-glycosylated region with up to 16 occupied O-glycosylation sites within the peptide sequence Thr82-Ser121.

Additionally, all the six N-glycosylation sites were identified and five of them were characterized in a site-specific manner. Relative quantitation of the glycopeptide N-glycoform distribution largely overlapped with the total

released N-glycan profile analyzed by MALDI-TOF/TOF-MS/MS.

This data can form the basis for designing further functional studies on C1-Inh glycosylation.

(245) Enabling Tools for Protist Pathogen Glycobiology

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Protozoan parasites plague tens of millions of humans and drugs are limited, so new targets for control are urgently needed. A considerable literature implicates glycosylation-related processes in the biology, virulence and persistence of two well-known parasites: *Trypanosoma cruzi*, agent of Chagas disease, and *Toxoplasma gondii*, responsible for toxoplasmosis. The advent of CRISPR/Cas9 genome editing enables incisive tests of the proposed roles of glycans genetically. Nevertheless, glycobiology is a specialty area imposing to non-aficionados. We are developing a resource for parasitologists that i) delineates the framework of glycogenes contributing to the assembly of parasite glycans, ii) provides an easy-to-implement protocol for their disruption and, iii) for select examples, documents the feasibility of disruption and consequences on the cellular glycome. We describe here a web-based guide RNA selection tool for multiple parasites. An existing method that electroporates gRNAs into Cas9-expressing *T. cruzi* epimastigotes has the benefit of efficient, rapid gene editing but suffers from Cas9 toxicity. We show here how use of "buffering" gRNA reduces toxicity and electroporation of Cas9/gRNA complexes eliminates it altogether while providing highly efficient KOs in any *T. cruzi* strain, as well as in *Trypanosoma brucei*. As an orthogonal approach to evaluate contributions to fitness, populations transfected with a library of glycogene gDNAs will be sequenced for gDNA persistence or attrition. Glycogenes of *T. gondii* are being targeted systematically using double-CRISPR/Cas9 plasmids and a transient generic (no homology ends) floxed-DHFR-resistance amplicon that inserts into the gRNA-directed Cas9-mediated double strand cut site. This strategy has high efficiency, avoids persistence of toxic Cas9, ensures glycogene disruption, is amenable to multiple rounds of disruption, and is applicable to wild-type strains with intact non-homologous end joining. To assess glycomic consequences, we are consolidating protocols for sample preparation, glycan release, permethylation, and LC and direct infusion MS/MS analysis, and generating standards, to examine N- and O-glycans, phosphodiester-linked glycans, GPI-anchors, GIPLs, and other glycolipids. We are expanding existing our GRITS database to semi-automate the analyses. With this toolbox, we envision that non-specialists can make connections between their favorite functions and the cellular glycosylation machinery. This work is supported by NIH 1R21-AI123161 (Common Fund).

(246) A High Throughput and High Resolution Glycan Analysis Platform on Applied Biosystems Multi-Capillary CE

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Glycosylation is one of the key post-translational modifications of proteins and for therapeutic proteins. It affects stability, immunogenicity, function, pharmacokinetics and pharmacodynamics. In therapeutic proteins, N-linked glycans may display macro- and micro-heterogeneity. The degree of this variation can be due to cell line, media/feeds, and process. Because of the importance of glycosylation in structure and function of therapeutic proteins, it is necessary to accurately detect and quantitate the changes. Glycan profiling begins at cell culture development and continues through process development and quality control.

We have developed an integrated glycan analysis platform consisting of magnetic bead based sample prep kits with multiple dyes for glycan labeling, multi-capillary CE instrument for parallel analysis of labeled glycans and assay specific software for glycan quantitation. For glycan labeling, along with conventional APTS dye, two new rapidly reacting fluorescent dyes were developed. Glycan cleavage, dye labeling and excess dye removal steps were streamlined on a magnetic bead platform. We eliminated the toxic sodium cynaoborohydride chemistry and vacuum centrifugation steps. N-glycans from 96 samples can be processed with <3 hours of hands-on time with analyzed data from 96 samples in 7-9 hours.

Here, we report repeatability data of labeled glycan samples analyzed over a period of 7 months showing sample stability and opportunity for re-analysis of original samples. Furthermore, injection repeatability of 300 injections for all three dyes shows reproducibility and robustness of the capillary array. Lastly, our newly developed software, which is user friendly and easy to use, can analyze small to large data sets in less than one hour, providing glycan profiles, relative quantities, %CV, and trending of relative quantities of specific glycans of samples from different conditions. Our fully integrated solution of easy sample prep, high throughput instrumentation and glycan assay specific software can offer a platform for rapid N-glycan analysis and quantitation.

(247) Revolutionary Streamlined and Rapid N-Glycan Preparation Directly from IgG in Cell Culture

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In bioprocess development of biotherapeutics, quick and accurate glycan profiling would give an advantage to monitor the characteristics of the glycoforms toward the successful production that is essential for maximizing their efficacies. Glycan preparations from IgG, in general, require several processes, e.g. antibody purification from cell culture supernatant, glycan release from antibody, glycan purification and glycan derivatization, taking hours to days to complete the total operations. Thus, those conventional approaches are either laborious, time-consuming, or unwelcome due to use of a toxic reagent. To aid researchers in the field, we have developed an unparalleled solution for N-glycan sample preparation from IgG and Fc-fusion proteins, EZGlyco™ mAb-N Kit with 2-AB. For the development of the novel product, we tried to shorten and integrate the processes employing a single microcentrifugal device to provide a streamlined manipulation of N-glycan sample preparation.

With the EZGlyco™ mAb-N Kit with 2-AB, N-glycan preparation from IgG and Fc-fusion proteins can be executed from various sample sources such as cell culture supernatant in upstream development for clonal selection and media optimization and further along in the biological process for downstream characterization. The Kit has ingeniously condensed the multiple steps in the sample preparation ranging from purification of IgG in culture supernatant to HPLC-ready labeled N-glycans, circumventing multiple sample transfers and drying steps and bypassing a separate IgG purification step. The streamlined and rapid procedure enables researcher to complete N-glycan preparation in less than two hours with accelerated PNGase F digestion with S-BIO's proprietary Glycan Release Enhancer Solution and a non-toxic reducing agent for 2-aminobenzamide (2-AB) labeling with no drying step needed during sample preparation. The Kit is currently designed for one of the most frequently employed labels, 2-aminobenzamide (2-AB), thus making it advantageous for utilizing an available vast database for 2-AB-labeled glycans.

The study presents method comparison and steps for EZGlyco mAb-N Kit with 2-AB, which enables a rapid, robust and reliable N-glycan analysis for IgG and Fc-fusion glycoproteins.

(248) New Tool to Study Mucin-Type O-glycosylation Using a Bump-Hole Strategy: Exploring an Orthogonal Polypeptide GalNAc-Transferase T2 and UDP-Sugar Pair

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O-Glycosylation is one of the most abundant forms of post-translational modifications, which plays an important role in regulating protein functions. Unlike other types of protein O-glycosylation, understanding of mucin-type O-glycosylation is highly limited due to the complex structure of mucin-type glycopeptides and the lack of readily accessible tools to identify O-glycoproteins or sites of O-glycosylation. Mucin-type O-glycosylation is initiated by twenty distinct polypeptide GalNAc-transferases (ppGalNAcTs), which transfer a GalNAc residue from UDP-GalNAc to serine or threonine residues of target proteins. Because of the functional redundancy and overlapping target sites among the twenty ppGalNAcTs, identifying the cellular function of these enzymes still remains a significant challenge.

It has been revealed that one member of ppGalNAcT family, ppGalNAcT2, modulates proprotein convertase activation, and improper O-glycosylation results defective proteolysis. Although a substantial amount of effort has been made to investigate the role of ppGalNAcT2, the understanding of its function is still limited. Therefore, we endeavored to develop a novel tool to discover substrates for ppGalNAcT2. To find the target of ppGalNAcT2, we rationally designed an orthogonal ppGalNAcT2 and UDP-GalNAc analog pair using a bump-hole strategy. Herein, we report that an engineered ppGalNAcT2 can selectively transfer a GalNAc analog bearing a bioorthogonal tag to an acceptor protein, which enables identification of the substrate for ppGalNAcT2 by exploring metabolically labeled glycopeptides. Moreover, this approach can be applied to other families of ppGalNAcTs, and it will help to decipher the role of each ppGalNAcT in cellular function.

(249) Comprehensive analysis of protein glycosylation from prostate cancer cells using automated methods to release glycans and glycosite-containing peptides

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Abstract: Glycosylation is one of the most common modifications of proteins, however, due to the complexity and heterogeneity of glycoprotein glycoforms, established techniques have focused on the analysis of de-glycosylated glycopeptides or released glycans, separately. Recently, we have developed a chemoenzymatic method called solid phase extraction of N-linked glycans and glycosite-containing peptides (NGAG) that facilitates a comprehensive analysis of the N-linked glycoproteome. With the advancement of mass spectrometry instrumentation, efforts have shifted to the development of automated sample preparation methods that emphasize high-throughput and reproducibility. Towards this goal, we have adapted the NGAG method into a tip-based format (NGAG-Tip) that simplifies the integrated workflow of isolation of N-linked glycans and glycosite-containing peptides from biological samples. The automated methodology was first optimized using bovine fetuin as a standard glycoprotein, and then applied to investigate changes in N-linked glycosite and N-linked glycan levels following increased alpha (1,6) fucosyltransferase (FUT8) expression in prostate cancer cells. We employed Stable

Isotope Labeling of Amino Acids in Cell Culture (SILAC) to quantitatively assess protein expression changes between LNCaP and LNCaP-FUT8 transformed prostate cancer cells in different cellular components. ESI-LC-MS/MS analysis revealed alterations in global protein expression and N-linked glycosite occupancy, and MALDI-TOF-TOF analysis identified a subsequent increase in core-fucosylated N-linked glycan branching. In this study, we demonstrated the use of automated NGAG approach could significantly reduce sample processing time and yield excellent reproducibility to comprehensively investigate the impact of FUT8 expression on the N-linked glycoproteome in prostate cancer cells. The automated methodology can have applications in high-throughput sample preparation in combination with liquid handling robotic systems for the comprehensive analysis of the N-linked glycoproteome in a variety of biological samples.

(250) Glycan Microarrays and Glycomics Services through the National Center for Functional Glycomics and the Harvard Medical School Center for Glycosciences

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The National Center for Functional Glycomics (NCFG) at Beth Israel Deaconess Medical Center at Harvard Medical School is one of four Glycomics-related Biomedical Technology Resource Centers in the U.S., funded by the National Institutes of Health. 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." We have 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. The paramount service offered 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 variety of defined glycan arrays, including mannose-6-phosphate, modified sialic acids, and glycopeptides. This number of glycan arrays is continuously growing, and will soon include 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 given 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 microarrays are continually being developed, and requests for shotgun microarrays of new materials can be directed to the NCFG. We also offer various glycomic analyses using MALDI-TOF and LC-MS. The specific focus of the NCFG hinges on technology development in the glycosciences, emphasizing the molecular mechanisms of glycan recognition by proteins important in human biology and disease, and we aim to support the community in their endeavors into defining protein-glycan interactions. This presentation will show some of the exciting new results generated through the NCFG in analyses of GBPs on various glycan microarrays at the NCFG. http://ncfg.hms.harvard.edu/home *P41GM103694*.

(251) Direct Characterization of the Maize Starch Synthase IIa Product Shows Maltodextrin Elongation Occurs at the Non-Reducing End

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A comprehensive description of starch biosynthesis and granule assembly remains undefined despite the central nature of starch as an energy storage molecule in plants and as a fundamental calorie source for many animals. Multiple theories regarding the starch synthase (SS)-catalyzed assembly of (α1-4)-linked D-glucose molecules into maltodextrins generally agree that elongation occurs at the non-reducing terminus based on the degradation of radiolabeled maltodextrins though recent reports challenge this hypothesis. Surprisingly, a direct analysis of the SS catalytic product has not been reported to our knowledge. We expressed and characterized recombinant Zea mays SSIIa and prepared pure ADP-[13CU]-glucose in a one-pot enzymatic synthesis to address the polarity of maltodextrin chain elongation. We synthesized maltoheptaose (DP7) using ADP-[13CU]-glucose, maltohexaose (DP6) and SSIIa. Product analysis by ESI-MS revealed the [13CU]-glucose unit was added to the non-reducing end of the growing chain, and SSIIa demonstrated a >9000-fold preference for addition to the non-reducing end versus the reducing end. Independent analysis of [13CU]-glucose added to maltohexaose by SSIIa using solution NMR spectroscopy confirmed the polarity of maltodextrin chain elongation.

(252) A New Method for Determining Polysialic acid Chain Length

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Polysialic acid (polySia) is an anionic sugar polymer that can consist of 8 to 400 monomers of sialic acid. This glycan modification has been identified on less than ten proteins while the protein neural cell adhesion molecule (NCAM) is the most common substrate for polySia. In vertebrates, PolySia is added to protein substrates in the Golgi by two polysialyltransferases, St8Sia II (STX) and St8Sia IV (PST), in a processive manner to both N- and O-linked glycans. Recent work studying polySia importance in human embryonic stem cell (hESC) differentiation concluded that polySia is critical for specification of cell lineage—where PST is expressed in endoderm and mesoderm lines and STX in ectoderm lines. Furthermore, PST expression is essential for differentiation into the endoderm lineage. In addition, polySia is known to bind growth factors (e.g. Fibroblast Growth Factor 2 and Brain-derived neurotrophic factor) where its chain length is a crucial determinate for binding. Thus, understanding the regulation of chain length could give insight into how a cell regulates signaling molecules using polySia. Our goal is to further understand the role of polySia in hESC differentiation; specifically, how polySia chain length is regulated/involved. To achieve this goal, we have chosen two hESC derived cell lines that are both polySia and NCAM positive. Neural crest, STX expressing, and heart epicardium, PST expressing, chain lengths were measured and compared. To measure chain length of polySia, pronase-digested glycopeptides prepared from cell lysates were fractionated on a strong anion exchange column, then sialic acid in each fraction was determined by DMB-labeling and detecting fluorescence. By fractionating samples before DMB labeling, intramolecular hydrolysis of polySia of longer chain lengths was reduced. Our study will help us to understand how chain length is regulated during hESC differentiation and its importance in cell signaling.

(253) Site-directed glycosylation of peptide/protein with homogeneous O-linked eukaryotic N-glycans

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Here we report a facile and efficient method for site-directed glycosylation of peptide/protein. The method contains two sequential steps: generation of a GlcNAc-O-peptide/protein, and subsequent ligation of a eukaryotic *N*-glycan to the GlcNAc moiety. A pharmaceutical peptide, glucagon-like peptide-1 (GLP-1), and a model protein, bovine α-crystallin, were successfully glycosylated using such an approach. It was shown that the GLP-1 with O-linked *N*-glycan kept unchanged secondary structure after glycosylation, suggesting the potential application of this approach for peptide/protein drug production.In summary, the approach provides a general platform for producing homogenous peptides/glycoproteins with O-linked eukaryotic *N*-glycans in a site-directed manner, which may contribute to the enhancement of therapeutic efficiency of modified peptides/proteins.

(254) Unraveling the complex regulation of glycosylation using a systems approach

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Protein glycosylation is fundamentally important to most biological processes. However, the diversity and complexity of glycosylation has made it difficult to systematically and comprehensively study the molecular mechanisms regulating glycan synthesis in vivo. However, we have recently developed a computational platform using probabilistic Markov modeling of glycosylation, and demonstrate that this approach can unravel how variations in nutrients and gene regulation impact the glycans presented by a cell. Specifically, our computational approach first leverages known biochemical preferences to enumerate the range of possible glycans that can be synthesized. Next, glycoprofiling and metabolomics data are used from control samples to train the model and quantify how glycosyltransferases and metabolic pathways contribute to glycan synthesis under normal conditions. Subsequently, this model is used as a platform to analyze additional omics data from samples with altered glycosylation. These analyses identify genes involved in glycosylation and metabolism that underlie changes in glycosylation, and suggest mechanisms by which glycosylation is regulated. We demonstrate here how this platform can be used to unravel the mechanisms underlying altered glycan synthesis in human tissues, to predict the organization of glycosyltransferases in the endomembrane system, and to guide glycoengineering for biopharmaceutical development. Thus, despite the complexity of glycosylation, novel systems biology approaches now provide tools to unravel its regulation.

(255) Novel Citronellyl-Based Photoprobes Designed to Identify ER Proteins Interacting with Dolichyl Phosphate and Dolichol-Linked Saccharide Intermediates in Yeast and Mammalian Cells

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Dolichyl phosphate (Dol-P)-linked mono- and oligosaccharide intermediates (DLOs) function as essential lipophilic glycosyl donors in protein N-glycosylation, C- and O-mannosylation and GPI anchor biosynthesis by providing a mechanism to ferry biosynthetic intermediates across the endoplasmic reticulum (ER) membrane into the lumen of the ER. Most of the ER proteins involved in the biosynthesis of DLOs are known. However, ER proteins mediating the transbilayer movement (flip-flopping) of the lipid intermediates from the site of synthesis on the cytosolic monolayer to the lumenal monolayer where mature DLO synthesis is completed, and to recycle the Dol-P formed from Dol-P-P released during the primary N-glycosylation reaction remain to be identified. We have previously developed a method to assay "flippase" activity by measuring the transport of water-soluble Man-P-Dol and Glc-P-Dol analogues into sealed, rightside-out ER vesicles. These water-soluble compounds contain a short-chain (C10) isoprenoid monophosphate, citronellyl phosphate (Cit-P), with a saturated a-isoprene unit characteristic of dolichols. The analogues have been modified by the addition of a photo-active benzophenone unit, equipped with a propyl alkyne "click chemistry" handle, to utilize as tools for identifying currently unknown Dol-P interacting ER proteins involved in the transbilayer movement of dolichol-linked intermediates. *In vitro* enzymatic assays with CHO MPDS were used to establish that the photoprobes contain the critical structural features recognized by pertinent enzymes in the dolichol pathway. ER proteins that photoreacted with the novel probes were coupled to biotin-azide by Cu (I) catalyzed azide-alkyne cycloaddition, captured onto streptavidin beads and identified by MS analysis after on-column trypsinization. These newly-designed photoprobes provide promising new tools for the identification of yet to be identified Dol-P, Man-P-Dol and Glc-P-Dol interacting ER proteins in yeast and mammalian cells.

(256) Human milk oligosaccharides early in life modulate and program intestinal microbiota and immunity in an autoimmune mice model.

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Objective: Early life nutrition such as breast milk is known to be an independent protective factor against development of type 1 diabetes. Human Milk OligoSaccharides (HMOS) are important bioactive components of human milk. The effect of early supplementation with HMOS on the autoimmune diabetes incidence in Non-obese diabetic (NOD/ShiLtJ) mice was evaluated and correlated to the protective effect with regulation of immune responses and modulation of gut microbiota.

Methods: NOD mice were fed diet containing human milk derived oligosaccharides from week 4 to 10 or normal diet. Diabetes incidence was determined by urine glucose tests. Pancreatic insulitis was characterized histologically. Naturally occurring regulatory T cell and T helper cell frequencies in the spleen were analyzed by flow cytometry. Intestinal microbiome composition was analyzed by 16S rRNA amplicons derived from fecal sample. Short chain fatty acids (SCFAs) were measured in cecal and fecal samples during intervention and diabetes development.

Results: Early supplementation with HMOS significantly reduced the incidence of diabetes up to the age of 30 weeks ($p < 0.05$). Suppressive effects were corroborated by lower pancreatic insulitis, lower Th1 induction and decreased T-cell activation markers (CD25 and CD69) expression in the spleen, although Th17 cells remained unchanged. Spleen regulatory T cells (CD4+CD25+FOXP3+) were reduced in the HMOS. Total cecal and fecal SCFAs were elevated by HMOS diet, and significantly different during intervention, resulting in increased levels of SCFAs known to be immune modulatory. Moreover, the composition of intestinal microbiota was found significantly different between the groups.

Conclusion: Temporary dietary exposure of NOD mice to HMOS in early life reduced the incidence of autoimmune diabetes beyond the intervention period. These results implicate that human milk derived oligosaccharides modulates the immune development and microbiome composition leading to suppression of spontaneous autoimmune development later in life.

(257) Dissecting the Unique Features of Neutrophil Glycobiology in Inflammation and Infection using Glycoanalytics

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The granulated neutrophils are front-line immune cells critical for the function of the innate immune system. Many aspects of the structure and function of the heterogeneous neutrophil glycoproteome which shows strong subcellular-specific characteristics remain unresolved. Enabled by technology advancements in glycomics and glycoproteomics, we here investigate the intriguing protein *N*-glycosylation of human neutrophils in the context of inflammation and bacterial infection. An under-reported class of truncated *N*-glycoproteins, paucimannosidic proteins, was initially discovered in sputum from pathogen-infected human lungs (Venkatakrishnan *et al.*, *Glycobiology*, 25(1):88, 2015). Their structures displaying simple monosaccharide compositions i.e. $Man_{1-3}GlcNAc_2Fuc_{0-1}$, the associated biosynthetic

machinery involving maturation stage-specific expression of β-hexosaminidases and their preferential subcellular location in azurophilic granules of pulmonary neutrophils were demonstrated (Thaysen-Andersen *et al.*, *J Biol Chem*, 290 (14):8789, 2015). Importantly, these compartment- and inflammation-associated glyco-signatures were present on intact bioactive proteins including cathepsin G, azurocidin and neutrophil elastase indicating that they are not degradation products (Loke *et al.*, *Biomolecules*, 5(3):1832, 2015). Recent glycomics-based studies on isolated granules of human neutrophils confirmed that paucimannosylation is enriched on proteins residing in the azurophilic granules, but also present in other compartments. In addition, paucimannosidic proteins were shown to be preferentially secreted, but not incorporated into the plasma membrane above constitutive levels, upon inoculation of isolated neutrophils with *P. aeruginosa* and other virulent stimuli thereby confirming granular mobility and suggesting extracellular functions of paucimannosidic proteins. Preliminary data shows that isolated paucimannosidic proteins displayed affinities to mannose-binding lectin and showed bacteriostatic activities towards virulent *P. aeruginosa* supporting immune-related functions of paucimannosylation in activated human neutrophils. Interestingly, isolated neutrophils from a Sandhoff disease patient displaying a *HEXB*$^{-/-}$ genotype showed reduced protein paucimannosylation relative to an age-paired healthy individual thereby confirming the importance of β-hexosaminidases in the biosynthesis of paucimannosidic proteins. Finally, other granulated immune cells were shown to also express paucimannosidic glycans indicating that these under-reported glycoproteins are integral to the innate immune system across multiple cell types. In conclusion, we here provide insights into the intriguing features of neutrophil glycobiology by expanding our knowledge of the structure, function and biosynthesis of the spatiotemporally-regulated protein paucimannosylation in the context of inflammation and infection.

(258) Sialylation is indispensable for establishment of fetal-maternal immune tolerance

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Sialylation is pivotal for murine embryonic development, as loss of the bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), which is essential for de novo sialic acid (Sia) synthesis, results in embryonic death. However the underlying mechanisms leading to fetal demise have never been elucidated. To investigate the role of Sia during embryonic development, we established a knock-out mouse of the Sia activating enzyme CMP-sialic acid synthase (CMAS). Like GNE knock-out animals, CMAS deficient mice (*Cmas*$^{-/-}$) showed embryonic lethality around embryonic day 9 (E9) and loss of sialylation. Analysis of embryonic development revealed intrauterine growth restriction (IUGR) from E7.5 and disturbed formation of the ectoplacental cone and the chorionic plate, both essential for placental development. Further investigation unveiled that *Cmas*$^{-/-}$ embryos also suffer from excessive deposition of basement membrane material at the Reichert's membrane, which is the outermost basal membrane and establishes fetal-maternal nutrient and waste exchange until the placenta is properly established. Moreover was the fetal-maternal interface of *Cmas*$^{-/-}$ animals significantly infiltrated with maternal neutrophils from E6.5 onwards and neutrophil infiltration increased with proceeding pregnancy. In addition, the cell surface of asialo trophoblasts, which were heavily α2,3 sialylated in control animals, exhibited deposition of the complement component C3, indicating a multifactorial inflammation of the asialo extraembryonic tissue. As fetal tissue, due to its paternal components, represents a semi-allograft to the mother, the maternal decidua has to provide an immunologically specialised environment and loss of fetal-maternal immune homeostasis is the major cause of pregnancy complications, such as recurrent fetal loss and preeclampsia. Therefore the immune modulatory function of Sia, e.g. via sialic acid binding Ig-like lectins (Siglec) and factor-H, might be of particular importance in maintaining the fetal-maternal immune tolerance.

(259) Identification, regulation and possible functions of newly identified polysialic acid carriers in microglia and macrophages

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Polysialic acid (polySia) has been implicated in modulating the activity of microglia, the innate immune cells of the brain. During brain development, polySia is mainly presented by the neural cell adhesion molecule NCAM on the surface of neurons. Recently, however, we discovered a pool of polySia in the Golgi compartment of NCAM-negative microglia and demonstrated that neuropilin-2 is one, but not the only polySia carrier in these cells (NRP2; Werneburg *et al.* 2015, Glia 63:1240). We therefore performed an unbiased glycoproteomic analysis and identified the E-selectin ligand-1 (ESL-1) as novel polySia protein carrier residing in the Golgi of stem cell-derived microglia and human THP-1 macrophages (Werneburg *et al.* 2016, Glia 64:1314). Comparing microglia from mice negative for either of the two the polysialyltransferases, ST8SIA2 and ST8SIA4, discloses that ESL-1 and NRP2 are polysialylated by ST8SIA4. As shown in organotypic brain slice cultures, Golgi-localized

polySia appears during injury-induced microglia activation and is lost in response to inflammatory stimulation by lipopolysaccharides (LPS). In cultured microglia and THP-1 macrophages, LPS causes cell surface translocation and a rapid release of the two polysialylated proteins. Addition of metalloproteinase inhibitors prevents polySia depletion, indicating that the release is mediated by protein ectodomain shedding. Furthermore, we demonstrate elevated levels of LPS-induced activation in ST8SIA4-negative microglia as well as the inhibition of nitric oxide and inflammatory cytokines by soluble bacterial polySia (colominic acid) or by polySia produced on an NCAM fragment in glycoengineered plants. Together these data demonstrate that polySia inhibits microglia activation independent of its protein carrier, and indicate that ectodomain shedding of polySia-ESL-1 and polySia-NRP2 constitutes a cell-intrinsic mechanism for a negative feedback regulation of LPS-induced activation of microglia and possibly macrophages. The potentially distinct mechanisms for shedding and responding to the two microglial polySia carriers are currently under investigation.

(260) Core-1 O-glycosylation is essential for B cell development and homing

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As the sole source of immunoglobulins, the development, migration, and function of B cells are critical in mediating immune responses against pathogenic insults, while dysregulation of these processes causes various B-cell-related disorders. Experiments have revealed that besides protein phosphorylation, other post-translational modifications act mechanistically to control the immune system. Here we have identified a key role for protein O-glycosylation as controlling B cell development and homing. Historically, high PNA lectin binding, which binds to the core 1 O-glycan, has been used as a marker for germinal center B cells for decades. However, as to whether the O-glycans on B cells regulate B cell biology has not been explored. The expression of core 1 O-glycans on all glycoproteins is regulated through the action of T-synthase and its molecular chaperone Cosmc, which is required for T-synthase folding and acquisition of activity. In mice in which Cosmc was specifically deleted in B cells, the lymphocyte homeostasis in the periphery was disturbed, and the immunoglobulins levels in the plasma and BCRs expression levels on B cells were significantly altered. Moreover, the development of Cosmc-null B cells was altered in the bone marrow. Furthermore, the distribution pattern of Cosmc-null B in bone marrow was modified. Finally, by adoptive transfer, we found that the homing of Cosmc-deficient B cell to the blood and spleen was comparable to WT B cells. By contrast, the distribution of Cosmc-null B cells to the peripheral lymphoid tissues was altered. Taken together, our data demonstrate for the first time that core-1 O-glycosylation is essential for B cell development and homing.

(261) Surface expression of B Cell Maturation Antigen is regulated by its own single N-glycan

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B cell maturation antigen (BCMA), which belongs to a tumor necrosis factor receptor (TNFR) family, is required for the survival of plasmablasts and plasma cells in normal physiological condition. In some tumor cells or inflammatory accessory cells, the expression of BCMA also can be detected. Through binding to its ligands, a proliferation-inducing ligand (APRIL) or B cell-activating factor (BAFF), trimerization of BCMA activates downstream signaling cascade and provides survival signaling for the reacted cells. In our study, we unexpectedly identified BCMA as a glycoprotein with a single N-glycosylation site with an aid of sugar alkynyl probes coupled with mass spectrometry. Afterwards, this novel finding was further proved by mutating the identified glycosite and the change of mobility in SDS-PAGE after PNGase F hydrolysis, changing the previous thought that BCMA was a non-glycosylated protein. We observed that ectopically expressed glycosylation site-mutated BCMA (BCMA-N42A) exhibited lower surface expression than wild type (WT) BCMA.By treating cells with cycloheximide (CHX) to inhibit protein synthesis, we observed that surface expression of BCMA in BCMA-N42A-expressing cells or PNGase F-treated BCMA-WT-expressing cells was significantly decreased. To our surprise, the reduced expression of non-glycosylated BCMA is linked with the increased generation of soluble BCMA in the culture medium. Consistently, increased surface BCMA was detected in both WT BCMA and BCMA-N42A-expressing cells after treating cells with inhibitors that block the release of BCMA from membrane. These results imply that the N-glycan of BCMA is required for the retention of BMCA on cell surface and absence of N-glycosylation is associated with secretion of BCMA.

(262) Extensive glycosylation of Anti-Citrullinated Protein Antibodies variable domains in rheumatoid arthritis

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Rheumatoid Arthritis (RA), a chronic autoimmune disease affecting 1% of the population, is characterized by (synovial) inflammation and the presence of autoantibodies. Anti-Citrullinated Protein Antibodies (ACPA) are highly specific for RA as around 70% of the RA patients are ACPA positive. In this study, we aimed to characterize ACPA molecules in detail to better understand ACPA mediated biological effects. Structural analyses by gel-filtration revealed that ACPA-IgG exhibit a higher molecular weight (MW) than conventional IgG molecules. This higher MW is explained by the presence of additional *N*-linked glycans in the antigen-binding fragment (Fab) of ACPA-IgG. So far, this size-shift is detected in all analysed RA patients with active disease. Interestingly, using UHPLC and LC-MS, Fab glycosylation is estimated to be present on 90% of ACPA-molecules, which is five times higher than on ACPA-depleted IgG isolated from the same patients. In addition, the amount of Fab glycosylation was even more pronounced in synovial fluid, the site of inflammation, than ACPA-IgG molecules isolated from blood. More intriguingly is the observation that ACPA Fab-linked glycans were highly sialylated and showed more abundant fucosylation compared to control IgG from the same patient. Together, our results suggest that ACPA may gain novel immunological properties by introducing the Fab glycans, such as the capacity to extend the half-life of the auto-antibodies or the ability to bind to specific lectins via the hyper sialylated Fab-linked glycans. Interestingly, the consensus *N*-glycosylation site was not germ-line encoded but introduced upon somatic hypermutation. In addition, ACPA-IgGs are of low avidity which might indicate that the ACPA B cell are selected for their Fab glycan rather than antigen binding. Therefore, these data indicate that ACPA-F(ab)-glycosylation can be directly involved in the selection and outgrowth of human auto-reactive B cell-responses, thereby contributing in the pathogenicity of the disease.

(263) Protein O-GlcNAcylation is crucial for B cell activation

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O-linked β-N-acetylglucosamine (*O*-GlcNAc) modification (*O*-GlcNAcylation) is a post-translational modification that adds a GlcNAc to serine or threonine residue of nuclear and cytosolic proteins. *O*-GlcNAcylation is catalyzed by *O*-linked N-acetylglucosaminyl transferase (OGT) and can be reversely removed by *O*-GlcNAcase (OGA). O-GlcNAcylation is involved in the regulation of many functions of proteins, including protein stability, localization and transcriptional activities and plays important roles in many diseases such as diabetes, neurodegenerative diseases and cancer. The functional role and molecular mode of action of protein *O*-GlcNAcylation in B cells is less implicated. We showed that protein *O*-GlcNAcylation accumulates after cross-linking of B cell receptor (BCR) by anti-IgM in mouse splenic B cells and that inhibition of OGA by a specific inhibitor, thiamet G, promotes anti-IgM-mediated activation of B cells. Comparative phosphoproteomic analysis reveled several *O*-GlcNAc-dependent phosphoproteins in B cell activation. Furthermore, we created a mouse line in which *Ogt* is deleted in a B cell-specific manner to demonstrate that *Ogt* ablation impairs BCR cross-linking induced cell activation and survival. Mice with B cell-specific deficiency in *Ogt* showed severe defects in the formation of germinal center and production of antigen-specific antibody following immunization. Crucial *O*-GlcNAcylated proteins in BCR signaling that controls B cell activation are identified.

(264) Identification of Siglec ligands using proximity labeling method

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Endogenous lectins play important roles in interpreting the information encoded in glycans. Siglecs are a family of receptor-type vertebrate lectins that recognize sialic acids and mediate signal transduction. Deciphering the molecular mechanisms of the Siglec-ligand interaction is essential to develop strategies to target this interaction. However, limited information regarding the identities of glycoproteins that serve as Siglec ligands is available at present, because of the technical difficulties in identifying such ligands due to inherently weak and transient nature of the interactions between Siglecs and their ligands.

To overcome this problem, we have developed a new method to identify Siglec ligands based on tyramide signal amplification principle. In brief, the cells expressing Siglec ligands are first incubated with FLAG-tagged recombinant Siglec protein coupled with peroxidase-conjugated anti-FLAG antibody (Siglec-peroxidase complex). The cells are then incubated with hydrogen peroxide and biotin-tyramide, which is converted to a short-lived radical species by peroxidase and covalently ligate to the proteins in the vicinity of Siglec-peroxidase complex. Biotinylated proteins are purified by streptavidin-magnetic beads and identified by mass spectrometry. As a proof of concept, we used recombinant CD22/Siglec-2 to probe its ligands on human B-lymphoma cell line, and identified several glycoproteins including CD45 and endogenous CD22/Siglec-2, both of which have been reported to be Siglec-2 ligands. The specificity of this labeling method was confirmed by sialidase treatment of target

cells and by using glycan recognition-deficient recombinant CD22/Siglec-2 mutant probe. We have applied this method to identify the potential ligands of other Siglecs, including Siglec-15. This method may be applicable for the identification of the ligands of other lectins.

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(265) Molecular Mechanisms for Carbohydrate Presentation to CD4+ T cells by MHCII Pathway

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Most bacterial surfaces are decorated with capsular polysaccharides (CPSs). These CPSs have unique structures and contribute to the bacterial virulence. Therefore, CPSs have been attractive candidates as vaccine components against bacterial diseases and have been used as the main components of glycoconjugate vaccines (composed of CPS covalently coupled to a carrier protein) for the past three decades. Glycoconjugate vaccines have been successful in generating protective immune responses in human populations; however, problems still persist. A major concern is their variable immune response in infants, elderly, and HIV positive individuals. Additionally, they are generated with poorly controlled conjugation chemistries, which result in inadequately characterized, heterogeneous, and variably immunogenic vaccines. Mechanisms by which the immune system interacts with the components of the vaccine need to be understood to design knowledge-based, new-generation glycoconjugate vaccines. Recently, we demonstrated a novel mechanism in which a glycoconjugate vaccine is taken up by antigen presenting cells and a carbohydrate epitope is presented on the surface via a major histocompatibility complex class II protein (MHCII). This presented epitope is then recognized by, thus stimulating, carbohydrate specific CD4+ T cells. This study demonstrated T cell recognition of carbohydrate epitopes, which had previously been considered T cell independent antigens. Our working hypothesis for this mechanism is that carbohydrates in their pure form do not bind with MHCII. Instead, they must be covalently linked with a carrier protein, which can be processed within the endolysosomes of the antigen presenting cell into smaller $glycan_p$-peptides, processed glycan ($glycan_p$) linked with carrier peptide, to be presented for T cell recognition. We utilize an in depth structural and biochemical approach to delineate the mechanisms of carbohydrate epitopes binding with MHCII. We employ a model bacterial polysaccharide antigen, CPS of type 3 *Streptococcus pneumoniae* (Pn3), conjugated to carrier protein or peptide. We have collected evidence for carbohydrate presentation on the surface of antigen presenting cells, carbohydrate epitopes binding with MHCII, and carbohydrate specific T cell recognition. This study will serve towards understanding glycoconjugate vaccine mechanisms and help in future knowledge based vaccine generation.

(266) The multifunctional human lectin galectin-3 is a glycosaminoglycan-binding protein

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Glycan-binding proteins belong to two distinct groups: lectins and glycosaminoglycan (GAG)-binding proteins (GAGBPs). Human beta-galactoside specific lectin galectin-3 (Gal-3), which has been shown to contribute to numerous biological events, is a member of the first group. GAGBPs, on the other hand, include a variety of convergently evolved proteins such as growth factors, cytokines, morphogens and extracellular matrix proteins. The general architecture of the binding sites of lectins, their preferred glycan structures and their mode of action are fundamentally different from those of GAGBPs. A member of one group rarely possesses the characteristics of both. Our data, however, show that Gal-3 is a notable exception to that rule. In the present study, we report several novel findings. First, calorimetric and spectroscopic data show for the first time that Gal-3 behaves like a GAGBP. The lectin interacts with unmodified sulfated GAGs (heparin and chondroitin sulfates) and chondroitin sulfate proteoglycans (CSPGs) at physiological pH and NaCl concentration. Second, the binding of sulfated GAGs and CSPGs to Gal-3 is not a result of non-specific interactions, rather sulfated GAGs and CSPGs specifically bind to Gal-3, most likely, via the LacNAc/lactose binding site of Gal-3. Finally, while heparin and chondroitin sulfate B (dermatan sulfate) are found to be monovalent ligands of Gal-3, chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) and bovine CSPG engage in multivalent binding with Gal-3. Such multivalent binding leads to the formation of noncovalent cross-linked complexes, which is reversible by lactose and GAGs. Hill plot analysis of calorimetric data reveals that the binding of CSA, CSC and bovine CSPG to Gal-3 is associated with progressive negative cooperativity effects. Identification of Gal-3 as a GAGBP should help reveal new functions of Gal-3 mediated by GAGs and proteoglycans [1].

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(267) Identification and purification of novel glycan-binding cytotoxic hemolysins that interact with cholesterol

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Lysins are cytotoxic proteins, peptides or glycosides that are capable of fragmenting mammalian cell membranes by specifically binding to membrane components. Certain lysins, called hemolysins, can disintegrate mammalian blood cells. A few of the reported lysins recognize specific glycan structures. We found, for the first time, a group of glycan-binding hemolysins (we named the group as Hemolysin-X or HelyX) from closely related species of flowering plants. Members of HelyX were isolated by ammonium sulfate precipitation, size exclusion chromatography and high performance liquid chromatography. Activity and ligand binding properties of HelyX were studied by plate based assays and spectroscopy. Interactions of HelyX with mammalian and fungal cells were documented through microscopy and flow cytometry. HelyX showed rapid and robust hemolytic activities even at a very low concentration. The molecular weights of these hemolysins range from 2 kDa to 10 kDa. Hemolysis by HelyX was completely inhibited by serum glycoproteins (Fetuin, asialofetuin, thyroglobulin and fibrinogen) and cholesterol. Interestingly, the hemolysins of cholera bacteria were reported to show similar binding specificities. When tested with colon and breast cancer cell lines, all the members of HelyX showed significant apoptotic activities. The hemolysins also arrested the growth of yeast cells.

(268) B cell independent sialylation of IgG

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The behavior of IgG can be functionally switched from pro- to anti-inflammatory by the action of the ST6Gal1, the only sialyltranferase which adds α2,6 sialic acids to the conserved N-glycans in Fc domain of IgG. While it is estimated that only 10% of the circulating IgG population is sialylated, this fraction is responsible for the anti-inflammatory properties of large-dose intravenous immunoglobulin (IVIg) therapy used to treat autoimmune disorders. However, the conditions under which sialylated antibodies are made and the regulation of ST6Gal1 in B cells driving this sialylation event remain to be revealed. Here, we investigate a conditional knock-out (cKO) mouse with a B cell-specific ablation of ST6Gal1, and determine that the IgG sialylation status in these cKO animals showed no difference from wild type mice. These data demonstrate that IgG sialylation can take place in the extracellular compartment, putatively in the bloodstream, independent of the B cell secretory machinery. Additionally, we show that secreted ST6Gal1 is highly produced by cells boarding the hepatic central veins, and that IgG sialylation is powered by serum-localized nucleotide sugar donor (CMP-sialic acid) that is, at least partially, derived from degranulating platelets. Thus, antibody-secreting cells do not exclusively control the sialylation-dependent anti-inflammatory function of IgG. Rather, IgG sialylation can be regulated by the liver and platelets through the respective release of ST6Gal1 and sugar donor into the cardiovascular circulation. These findings expose an extracellular pathway in which circulation-localized glycoproteins, such as IgG, can be dynamically sialylated independent from the canonical secretory pathway.

(269) Platelet releasate fuels circulatory ST6Gal1 activity to modulate plasma glycoprotein sialylation

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IgG class antibodies modified by an α2,6-linked sialic acid are the active component of the established anti-inflammatory therapy high-dose intravenous immunoglobulin (IVIg). ST6Gal1 is the only enzyme responsible for the addition of this sugar linkage to glycoproteins throughout the body. Our laboratory recently reported that IgG sialylation occurs independent of the B cell secretory pathway. Instead ST6Gal1 secreted from the liver sialylates IgG in the circulatory environment. Using a novel sialyltransferase activity assay, we show that this circulatory ST6Gal1 is enzymatically active, and that there is sufficient nucleotide-sugar donor (cytidine-monophospho-sialic acid, CMP-SA) in serum to fuel endogenous enzymatic activity. We investigated platelets as a source of donor, and observed that platelet releasate contains sufficient CMP-SA to power this reaction *in vitro*. Lastly, we analyzed IgG sialylation in serum taken from tomosyn-1$^{-/-}$ mice, which have a ~40% deficiency in platelet degranulation, and found a concomitant 40% *in vivo* decrease in serum IgG sialylation. **We conclude that CMP-SA derived from activated platelets fuels circulatory ST6Gal1 activity for the extracellular sialylation of IgG.** These data support a novel glycosylation model where IgG sialylation is modulated by liver-secreted, circulatory ST6Gal1 that is fueled by platelet release of CMP-SA for the dynamic modification of plasma glycoprotein sialylation.

(270) Survey of receptor interactions with a novel array of mycobacterial glycans

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An array of glycans representing many of the major sugar structures present on *Mycobacterium tuberculosis* has been probed with a panel of glycan-binding receptors from cells of the innate immune system. The results provide an overview of the multiple interactions between mycobacterial

glycans and receptors that mediate uptake and survival in macrophages, dendritic cells and sinusoidal endothelial cells.

The panel of 61 chemically defined glycans was created by solution-phase synthesis and each glycan was conjugated to bovine serum albumin through an amino-octyl spacer. The resulting neoglycoproteins were arrayed on glass slides. Soluble fragments representing the extracellular portions of the cell surface receptors were labelled directly with fluorophores or by complexing biotin-tagged versions with fluorescently labelled streptavidin. Fluorescently labelled proteins were detected directly or following addition of labelled secondary antibodies. For each receptor, the profile of glycans bound was consistent across different labelling and detection schemes. In most cases, the receptor fragments retained the natural oligomeric states of the intact receptors.

Multiple different endocytic receptors, including DC-SIGN, DC-SIGNR (L-SIGN), langerin, and the mannose receptor, interact with mannose-containing caps found on lipoarabinomannans. These receptors fall into two categories: those that bind preferentially to structures with exposed Manα1-2Man groups and those that bind less selectively to any mannose residues with exposed 3- and 4-hydroxyl groups. Many of the glycans on the array are ligands for overlapping sets of receptors, suggesting that there are multiple, redundant routes by which mycobacteria can enter cells. None of the mycobacterial glycans are ligands for the macrophage galactose receptor, which is the only endocytic receptor tested that does not bind to mannose. Two receptors with signalling capability interact with very different sets of mycobacterial glycans. Dectin-2 binds to many of the same targets as the mannose-binding endocytic receptors. In complete contrast, mincle binds exclusively to trehalose-containing structures, including the simple Glcα1-1Glc headgroup of trehalose dimycolate.

These results suggest that, amongst the wide variety of glycan structures present on mycobacterial surfaces, two key subsets are predominantly responsible for interactions with cells of the innate immune system.

(271) Analogs of mycobacterial glycolipids binding to the macrophage receptor mincle

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The macrophage lectin mincle is a receptor for trehalose dimycolate, an unusual glycolipid on the surface of *Mycobacterium tuberculosis*. Mincle binding to trehalose dimycolate initiates signalling that stimulates the macrophage to produce cytokines. This stimulation plays an important role in infection by mycobacteria and in the action of derivatives of mycobacteria as adjuvants. Previous structural analysis of the C-type carbohydrate-recognition domain of mincle showed the mechanism for binding of the two glucose residues that are linked α1,1 in the sugar headgroup of trehalose dimycolate but the mechanism of interaction with the lipid portion of the glycolipid has been the subject of speculation.

Using a combination of binding assays, structural analysis by X-ray crystallography and site-directed mutagenesis we now show the basis for enhanced binding of mincle to glycolipid ligands in which hydrophobic substituents are attached to the 6-hydroxyl groups of trehalose. Binding studies with synthetic acylated derivatives of trehalose that mimic trehalose dimycolate indicate that the apparent affinity of mincle for glycolipid ligands correlates with the size of the hydrophobic substituents rather than reflecting specific structural preference. Crystal structures and mutagenesis show that the acyl group attached to the 6-OH of one glucose residue of trehalose can interact with a relatively broad hydrophobic area on the surface of mincle and can take on multiple conformations. The acyl group attached to the 6-OH of the other glucose residue is unlikely to contact the surface of the protein. These results define a minimal target structure which can form the basis for design of stimulating ligands for mincle with adjuvant activity.

In addition, the crystal structure of an extended portion of the extracellular domain of mincle, beyond the carbohydrate recognition domain, provides insight into the part of the protein that is linked to the cell membrane, where it interacts with its signalling partner, and helps to define how the sugar binding sites are orientated at the macrophage surface for binding to pathogens.

(272) GLYCOMICS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE LUNG TISSUE

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Cell surface glycosylation changes in tissue and disease-specific fashions. Such changes can have important consequences, especially when the changes involve the expression of specific glycans that engage complementary glycan binding proteins on inflammatory cells. The engagement of Siglec-9, a glycan binding protein expressed on neutrophils, with its appropriate glycan ligand has been implicated in the pathology of Chronic Obstructive Pulmonary Disease (COPD). In this study, we analyzed N-glycan, O-glycan and glycosphingolipid (GSL) glycans derived from COPD patient lung tissues. Two patient groups were selected based on disease severity as COPD (<50% FEV1) and control (>=80% FEV1). In each of these groups, glycan expression was assessed in upper lung tissue (Upper lobe) or lower lung tissue (Middle and Lower lobe). For O-glycan analysis, we employed our recently optimized mass spectrometric

methods (MS) that simultaneously enhance detection for neutral, sialylated, and sulfated O-glycans. We detected significant increases in sialylation in COPD patient lung tissue compared with control groups. Sulfated glycans that may be endogenous ligands for Siglec-9 (e.g.: 6 Sulfo sialyl lewis x) were also increased in COPD patient lung tissue. To confirm our MS results by an orthogonal approach, glycoprotein expression levels were also analyzed by lectin blotting using recombinant Siglec-9 Fc as a probe. Siglec-9 blotting showed that a band of high molecular weight was detected in COPD patient but not in control lung tissue consistent with MS analysis. GSL glycans were also profiled in COPD and control lung tissues. No significant changes in gangliosides were detected in COPD, although Lactosylceramide was slightly decreased in lower lung. Taken together, protein glycosylation in COPD lung tissue is shifted toward the induction of higher levels of sialylation and presentation of putative Siglec-9 ligands, perhaps representing compensatory responses aimed at reducing the neutrophilia associated with disease progression.

(273) ST3Gal1 truncates O-glycans and augments galectin-3 binding to CD45 in human B cells

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Lymphocyte activation is commonly accompanied by structural remodeling of glycans on membrane glycoproteins. On T cells, activation and differentiation lead to alterations in glycosylation that influence binding of endogenous lectins, including regulators of T cell survival (galectins) and trafficking (selectins). Based on these studies, we hypothesized B cells similarly undergo glycan restructuring during activation and differentiation that modulates endogenous lectin binding. To test this, we profiled human tonsil naive, germinal center (GC), memory, and plasmablast (PB) B cells by flow cytometry and immunohistochemistry with plant lectins. We found GC B cells show 4-fold increased expression of polylactosamine (polyLN) compared to naive, memory, and PB B cells, as measured by STA and LEA lectin binding. We further observed that GC B cells display more asialylated Core 1 O-glycans (PNA binding), consistent with numerous reports. Because polyLN and Core 1 sialylation can be simultaneously controlled by the Core 1 α2,3 sialyltransferase ST3Gal1 in T cells, we sought to test whether decreased ST3Gal1 expression may explain augmented polyLN levels in GC B cells. Indeed, we found that GC B cells express 4-fold less ST3Gal1 than naive and memory B cells. Furthermore, we observed that enforced ST3Gal1 expression in GC-like Ramos B cells ablates Core 2 polyLNs in both flow cytometric and MALDI-TOF MS analyses. As polyLNs are high affinity ligands for galectin-3 (Gal-3), a regulator of B lymphoma cell apoptosis, we tested whether GC B cells demonstrate increased Gal-3 binding. Unexpectedly, GC B cells bound significantly less Gal-3 compared to naive (4-fold) and memory (2-fold) B cells, and ST3Gal1 overexpression in Ramos B cells enhanced Gal-3 binding by >10-fold. Co-immunoprecipitation studies revealed the primary glycoprotein receptor of Gal-3 on Naive B cells to be CD45. Collectively, these studies implicate ST3Gal1 as a regulator of O-glycan polyLN and Gal-3 binding to B cells, primarily to CD45. Ongoing studies are focused on understanding the mechanism of altered Gal-3 binding, and how Gal-3 may influence CD45 activity. Because CD45 is a major receptor tyrosine phosphatase central to B cell receptor signaling, altered Gal-3/CD45 dynamics may have important consequences for development of high-affinity antibody responses in GCs.

(274) Characterization of IgG glycosylation in rheumatoid arthritis patients by MALDI-TOF-MSn and Capillary Electrophoresis

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Rheumatoid arthritis(RA) is a multisystem disorder in which immunological abnormalities result in symmetrical joint inflammation, articular erosions and extra-articular involvement. One of the most characteristic featuresof RA is activation of the immune system and production of rheumatoid factor, i.e.immunoglobulin M(IgM)and immunoglobulin G (IgG)antibodies directed against own IgG. It has recently been suggested that IgG glycosylation defectmay be involved in RA immunopathogenesis.

IgG glycosylation defectinvolved in RAhas been investigated byvarious methods,such as lectinbindingand immunosorbent ELISA assay.However, specific structures such as branching patterns of IgGN-glycans in RA have not been provided simultaneously with the quantification information. The complete characterization of glycans,including both qualitative and quantitative analysis,requires a combination of different methods.Multistage mass spectrometry with collision-induced dissociation (MSn)is a promising waytoassign individualglycan structuresas it can produce multiple-level fragments of a glycan through consecutive

fragmentations. In addition, capillary electrophoresis (CE) is an excellentmethod for resolution and quantification of glycans in virtue of high sensitivity, short analysis time and high resolving power for complex mixtures. Therefore, MSnand CE are a good combination for characterization of glycans.

In the present study, N-glycosylation of IgG inRA patients and in healthy people wascharacterized through identification of the released glycans using MSn.N-Glycosylation levels were determined through quantitation of the glycans byCE.Assignment of the IgGN-glycan structures was made through branching patternanalysisby MSn. Using CE,we were able to resolve and accurately quantify glycans and corroboratethat galactosylation and sialylation of IgG N-glycans in RA-cases were significantly lower than those in healthy subjects. Abnormal glycosylation of IgG in RA patients including defect of galactosylation and decrease of sialylation compared to healthy people is a useful marker for diagnosis and assessment of therapy effectiveness.

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(275) Different Airway Ligands for Human and Mouse Eosinophilic Siglecs

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Airway inflammation is driven by infiltration of innate immune cells. Siglecs (sialic acid binding Ig-like lectins) are regulatory molecules expressed on subsets of immune cells where most inhibit inflammation. Murine Siglec-F and human Siglec-8 are functional paralogs, both expressed on eosinophils. When Siglec-F or Siglec-8 on activated eosinophils binds to sialoglycan ligands on tissues, apoptosis is induced. On glycan arrays, both siglecs bind to the same sulfated sialylated glycan: Neu5Ac α2-3 6-SO4-Gal β1-4 GlcNAc (6'Su-SLacNAc). Despite their similarities, we report that their endogenous ligands diverged in both cell distribution and structures. Overlay of histological sections of human trachea with Siglec-8-Fc to locate siglec ligands revealed intense staining of submucosal serous cells and hyaline cartilage. In contrast, Siglec-F-Fc bound to human airway epithelium, mucosal and submucosal connective tissue and only slightly to cartilage. Siglec-8-Fc failed to bind to mouse trachea sections at all, whereas Siglec-F-Fc bound strongly to mouse airway epithelium, less so to mucosal and submucosal connective tissue and not to cartilage. In blots of electrophoresis-resolved extracts, Siglec-8-Fc ligands were not expressed on cultured human tracheal epithelial cells whereas Siglec-F-Fc bound to a ligand from cultured mouse tracheal epithelial cells previously identified as Muc5b (Kiwamoto *et al*, J Allergy Clin Immunol 135:1329, 2015). In extracts of postmortem human airways, Siglec-8-Fc bound to three size-classes of ligands in the range 270-1000 kDa which were purified and identified by proteomic mass spectrometry and/or Western blotting as the proteoglycan aggrecan. Chemical desulfation or sialic acid truncation eliminated Siglec-8-Fc binding, indicating sulfated sialylated structures. By comparison, Siglec-F ligands were unaltered in mice with mutated galactose sulfotransferases (Patnode *et al*, J Biol Chem 288, 26533, 2013). Aggrecan carries sialylated sulfated keratan sulfate chains. Treatment of human tracheal sections with keratanase or sialidase depleted Siglec-8-Fc binding whereas Siglec-F-Fc binding was increased after keratanase and eliminated by sialidase treatment. Although they are functional paralogs, and Siglec-F and Siglec-8 are both expressed on eosinophils where their ligation induces apoptosis, we conclude that they engage fundamentally different endogenous sialoglycan ligands. Supported by the Lung Inflammatory Disease Program of Excellence in Glycoscience (LIDPEG, P01HL107151, http://lidpeg.org).

(276) Ligands for siglecs in human airway exudates: comparison of Siglec-8, Siglec-9, Siglec E, and Siglec-F binding patterns

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Sialic acid binding Ig-like lectins (siglecs) are immune regulatory molecules expressed on distinct subsets of immune cells. Siglec-8 is found on human allergic inflammatory cells (eosinophils, mast cells and basophils), whereas its functional paralogue in mice, Siglec-F, is found on eosinophils, macrophages and microglia. Siglec-9 is found on human neutrophils, monocytes, dendritic and NK cells, and its orthologue in mice, Siglec-E, on the same cells except NK cells. Studies using human granulocytes and animal models indicate that engaging siglecs leads to inhibition of ongoing inflammatory responses through induction of immune cell apoptosis and/or inhibition of immune mediator release. To study native siglec counter-receptors in human airways, we collected

soluble exudates from postmortem human airways incubated separately in vitro for 24 h. High molecular weight soluble proteins released from airways were resolved by agarose-acrylamide composite gel electrophoresis, blotted, and probed with human-Fc chimeras of Siglec-8, Siglec-9, Siglec-E and Siglec-F. Exudates from trachea and bronchus contained multiple sialidase-sensitive high molecular weight siglec binding species (270 kDa to >4 MDa). These species co-migrated with bands from guanidinium chloride extracts of human trachea and lung parenchyma. Siglec-8-Fc bound to sialoglycans in the 270-1000 kDa range in tracheal exudates whereas Siglec-9 bound also to higher molecular weight species (>4MDa). Although Siglec-F is the functional paralog of Siglec-8, its binding to human exudate proteins revealed a pattern very similar to Siglec-9 binding and different from Siglec-8 binding. The Siglec-E binding pattern was distinct from the others, but retained binding to several glycoprotein molecules of sizes similar to those bound by Siglec-9. Unlike Siglec-F, Siglec-8 did not bind to any proteins in extracts of mouse trachea or cultured mouse tracheal epithelial cells. Siglec-9 ligand extracted from human trachea was purified and subjected to proteomic mass spectrometry, revealing MUC5B as the major protein. We conclude that exudates from human airways contain soluble ligands for siglecs, the binding pattern to these soluble ligands by Siglec-8 and Siglec-9 are distinct, and that the pattern of Siglec-F binding is similar to that of Siglec-9. Supported by the Lung Inflammatory Disease Program of Excellence in Glycoscience (http://lidpeg.org; P01HL107151).

(277) Galectin-8 stimulates a protective immune response in a viral infection model

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Galectin-8 is a mammalian lectin, endowed with pro-inflammatory properties. We have previously shown that Gal-8 is able to elicit antigen-specific CD4 T cell response in the transgenic DO11.10TCR$_{OVA}$ model, both *in vitro* and *in vivo*. Here, we further tested the stimulating capacity of Gal-8 in the elicitation of an effective immune response using an experimental foot and mouth disease virus (FMDV) model. For this purpose, soluble rGal-8 was co-administrated with antigen during immunization of BALBc/J mice with the inactivated virus (iFMVD). The use of a single dose of recombinant Gal-8 (rGal-8) in antigen formulation increased an specific and neutralizing humoral response, sufficient to enhance animal protection upon viral challenge. The response of rGal-8/iFMVD-immunized animals was also characterized by increased levels of IL-6 and INF-γ, as well as lymphoproliferation. These latter results were only observed at early times after immunization (48h), suggesting that Gal-8 participates in the elicitation of the adaptive immune response. In order to get a deeper insight in the mechanism by which Gal-8 stimulates the antigen-specific immune response, we investigated whether Gal-8 was able to promote antigen-presenting cells activation to sustain T cell activation after priming. Bone marrow-derived dendritic cells (DC) treated with exogenous rGal-8 exhibited a mature phenotype characterized by increased expression of the co-stimulatory molecules MHC II, CD80 and CD86 and the secretion of pro-inflammatory cytokines (IL-3, IL-2, IL-6, TNF, MCP-1 and MPC-5), and growth factor G-CSF. Of note, IL-6 was the most abundant cytokine induced in Gal-8-treated DC conditioned media. Remarkably, Gal-8-activated DC stimulated antigen-specific CD4 T cells more efficiently than their immature counterparts. Finally, the presence of Gal-8 increased antigen uptake by immature DC. Taking together, these findings strongly suggest that Gal-8 activates antigen-presenting cells to elicit a protective immune response, and argue in favour of the use of Gal-8 as an immune-stimulator molecule.

(278) GSnP-6, analogue of PSGL-1, inhibits P-selectin in vitro and in vivo

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P-selectin glycoprotein ligand-1 (PSGL-1) is a cell surface glycoprotein that is expressed on all leukocytes and endothelial cells, and supports leukocyte recruitment during inflammation and immune response. Detailed structural and mechanistic studies of PSGL-1 have revealed the presence of a recognition motif common to many glycosulfopeptide (GSP) that consists of clustered tyrosine sulfates (Tyr-SO3H) and a Core-2 *O*-glycan that bears a sialyl Lewis^x-containing hexasaccharide epitope (C2-*O*-sLe^x). Blockade of P-selectin and PSGL-1 interaction holds tremendous potential for the treatment of thrombosis, metabolic syndrome, cancer, Crohn's disease, and innate immunity disorders. Antibodies targeting P-sel/PSGL-1 interaction are challenging to

manufacture and limited by its shelf life, while small molecule inhibitors are limited by their low binding affinity and off target toxicity. Attempts of synthesizing PSGL-1 analogues remain difficult due to the poor region- and stereoselectivity during glycosylation, incompatible protecting groups for oligosaccharide synthesis, and poor stability of tyrosine sulfates. Here we report an efficient chemoenzymatic synthesis of glycosulfopeptides that mimic *N*- terminus of PSGL-1 and bind human P-selectin with nanomolar affinity (K_d ~ 22 nM). Key features of this synthesis involve the development of a scalable enantioselective synthesis of GalNac-Threonine and regioselective route to synthesize multigram C2-O-Glycan with appropriate protecting groups compatible to solid phase peptide synthesis. Furthermore, replacement of hydrolytically labile tyrosine sulfate with stable, isosteric sulfonates leads to a compound, termed GSnP-6, that is highly stable and blocks P-sel/PSGL-1 interaction both *in vitro* and *in vivo*.

(279) The Immunoglobulin G1 N-glycan Composition Affects Binding to each Low Affinity Fc? Receptor

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Immunoglobulin G1 (IgG1) is the major circulating human antibody and also the primary scaffold for therapeutic monoclonal antibodies (mAbs). The destruction of IgG-coated targets by cell-mediated pathways begins with an interaction between the IgG Fc region and multiple varieties of membrane-bound Fc γ receptors (FcγRs) on the surface of leucocytes. This interaction requires the presence of an asparagine-linked (N-)glycan on the Fc. Furthermore, changes to the N-glycan composition are known to affect the affinity of CD16A binding (an FcγR). Contemporary efforts to glycoengineer mAbs focus on increasing CD16A affinity and thus treatment efficacy but it is unclear how these changes affect affinity for the other FcγRs. Here we measure binding of the extracellular Fc-binding domains for human CD16A, B, CD32A, B, C and CD64 to six well-defined IgG1 Fc glycoforms to cover ~85% of the pool of human IgG1 Fc glycoforms. Core α1-6 fucosylation showed the greatest changes with CD16B (8.5-fold decrease), CD16A (3.9-fold decrease) and CD32B/C (1.8 fold decrease) but did not affect binding to CD32A. Adding galactose to the non-reducing termini of the complex-type, biantennary glycan increased affinity for all CD16s and 32s tested by 1.7-fold. Sialylation did not change the affinity of core-fucosylated Fc, but increased the affinity of afucosylated Fc slightly by an average of 1.16-fold for all CD16s and 32s tested. The effects of fucose and galactose modification are additive, suggesting the contributions of these residues to Fc γ receptor affinity are independent.

(280) New methods for assessments of clustered O-glycosylation and for determining the role of ST6GalNAc-II in the formation of galactose-deficient IgA1 in IgA nephropathy, an autoimmune disease

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IgA nephropathy (IgAN) is an autoimmune disease in which elevated levels of the autoantigen, galactose-deficient IgA1 (Gd-IgA1), are associated with disease progression. Galactose deficiency affects some of the clustered *O*-glycans in the IgA1 hinge region (HR). The HR *O*-glycans are added in a stepwise manner by glycosyltransferases in the Golgi apparatus. Glycosylation is initiated by the addition of GalNAc to Serine or Threonine by GalNAc transferases to form Tn antigen. Next, galactose may be added to GalNAc by C1GalT1 to form T antigen or sialic acid may be added to GalNAc by ST6GalNAc-transferases to form sialyl-Tn antigen. The sialyl-Tn antigen is considered a dead-end product that C1GalT1 cannot modify. However, the T antigen can be further modified through the addition of sialic acid on galactose by ST3Gal-I and/or on GalNAc by ST6GalNAc-transferases. Previous work has demonstrated that altered expression of key glycosyltransferase genes, including C1GALT1, COSMC (encoding C1GalT1 specific chaperone), and ST6GALNAC2 contributes to formation of Gd-IgA1 in IgA1-producing cells of patients with IgAN. Decreased expression and activity of C1GalT1 and Cosmc lead to increased formation of Gd-IgA1. Furthermore, increased activity of ST6GalNAc-II results in sialylated HR GalNAc and prevents subsequent galactosylation by C1GalT1, thereby promoting formation of Gd-IgA1. We have also shown that, *in vitro*, ST6GalNAc-II sialylates HR terminal GalNAc residues, even without galactose on any clustered HR *O*-glycans. It is unknown how galactose or sialyl-galactose attached to GalNAc may affect the activity of ST6GalNAc-II towards *O*-GalNAc of IgA1 HR. To better understand the glycosylation pathways involved in production of Gd-IgA1, we investigated the glycoform specificity of ST6GalNAc-II using liquid chromatography high-resolution mass spectrometry. We determined how ST6GalNAc-II adds sialic acid to an IgA1 myeloma protein, both with and without prior sialylation of galactose by ST3Gal-I. By quantifying relative abundances of the IgA1 glycoforms, we determined that the amounts of GalNAc, galactose, and sialic acid

attached to galactose play a role in the substrate preference of ST6GalNAc-II in a glycoform-specific manner. We will use these methods to further characterize the glycosylation pathways involved in production of Gd-IgA1 and determine the role of ST6GalNAc-II in formation of Gd-IgA1.

(281) Identification of the binding roles of terminal and internal glycan epitopes using enzymatically synthesized N-glycans containing tandem epitopes

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In nature, glycans play important roles in numerous biological events through binding with corresponding glycan binding proteins (GBPs). Usually, the carbohydrate moiety involved in these bindings is composed of the sugar residues at the non-reducing terminal by linking to each other in certain manners and forming specific epitope determinants. Nevertheless, internal epitopes may also participate in the binding and initiate subsequent signaling processes. To investigate the binding roles of terminal and internal epitopes in the glycan binding process, a focused library of asparagine-linked bi-antennary glycans with tandem epitopes including LacNAc (Galβ1,4-GlcNAc), sialyl LacNAc (Siaα2,3-LacNAc), 6-sialyl LacNAc (Siaα2,6-LacNAc), Lewis x (Galβ1,4-(Fucα1,3-)GlcNAc), sialyl Lewis x (Siaα2,3-Galβ1,4-(Fucα1,3-)GlcNAc), α-Gal (Galα1,3-LacNAc), and disialic acid (Siaα2,8-Sia), was synthesized herein via Core Isolation/Enzymatic Extension (CIEE) combined with one-pot multiple-enzyme (OPME) strategy. The produced glycans were separated by high performance liquid chromatography monitored with UV detector. In total, 36 glycans were prepared to milligram-scale, over 98% purity and were characterized by MS and NMR. Binding studies using selected GBPs and viruses demonstrated that when interpreting the binding between glycans and GBPs/viruses, not only the structure of the terminal glycan epitopes, but also the internal epitopes and/or modification of terminal epitopes needs to be taken into account.

(282) A Comprehensive N-glycan Microarray Reveals Glycan-Protein Binding Details

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As the entire complement of sugars, glycome may contain millions to structures, due to the inherit complexity and diversity of glycans. However, since the number of GTs in any genome and the natural connection between each sugar are limited,a new realization has mergedrecently that natural, biologically relevant humanglycome is limited to about 30K sugar sequences.Accessing these structures libraries of structurally well-defined glycans is prerequisite for understanding glycan-protein interactions. However, preparation of such libraries with large amounts from natural sources is tough as glycans found in nature possess inherited complexity, diversity and usually low abundance. We believe that Chemical Synthesis of core structures plus Enzymatic Extension to diversify the cores (CSEE) is a promising, practical and rapid strategy to ultimately overcome the complexity and diversity of glycans, yielding a Synthetic Glycome.

We have prepared around 100 structurally well-defined *N*-glycan structures via CSEE.[1,2] Each glycan only has one monosaccharide difference than others, providing a unique *N*-glycan library for probing glycan-protein binding details and impact of each monosaccharide on bindings. The *N*-glycans were then quantified and print as microarray with equal concentrations. Binding assay with lectins revealed detailed information of glycan protein bindings. For examples, lectin SNA specifically bindsα2,6linked sialic acid, and it was found that for bi-antennary *N*-glycans, the branch withoutα2,6Sia influences the binding in an orderly pattern. In addition, SNA has a stronger binding to α2,6Sia on α1,3Man branch, whereas MAL-I (lectin specifically bindα2,3Sia) prefers theα1,6Man branch. Further binding studies using virus hemagglutinins and glycan antibodies is undergoing.

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(283) Human Intelectin-1, a member of the X-type lectin family, binds specific microbial glycans

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The human body is constantly exposed to both pathogenic and commensal microbes. The innate immune system serves

as the first line of defense against pathogens and is indispensable for survival. Understanding the complexities and mechanisms of this innate immune system may facilitate the development of therapies to treat diseases caused by pathogens. Human intelectin-1 (hIntL-1) a member of the X-type family of lectins, is induced following pathogen infection as well as IL-13 stimulation, a cytokine generated by a TH2 response. By using recombinantly expressed hIntL-1 and glycan microarrays, we sought to elucidate the biological ligand (s) of hIntL-1. We engineered suspension-adapted HEK293T cells to secrete recombinant hIntL-1. Purification of hIntL-1 using β-galactofuranose affinity chromatography resulted in a disulfide-linked trimer as determined by SDS-PAGE. hIntL-1 was labeled with Alexa Fluor 488 and analyzed on the Consortium for Functional Glycomics (CFG) mammalian glycan array. Surprisingly, these results showed minimal hIntL-1 binding. We next tested the Alexa Fluor labeled hIntL-1 on the Microbial Glycan Microarray (MGM). Briefly, this array showed that hIntL-1 bound to specific cell surface microbial glycans containing either β-linked galactofuranose or glycerol phosphate. To support the MGM results, flow cytometry data confirmed hIntL-1 binding to only pathogens that tested positive on the array. Lastly, we utilized the LS174T cell line, a human goblet-like colon adenocarcinoma, to understand the signaling pathway(s) required for hIntL-1 induction. Following IL-13 stimulation, both the MAPK and JAK/STAT pathways were activated in LS174T. Pharmacologically inhibiting the MAPK pathway resulted in the loss of hIntL-1 induction. Our results support the role of hIntL-1 within the human immune system and possibly serving as a microbial detector molecule.

(284) Biochemical Characterization of Family GT47 Glycosyl Transferases Involved in Xylan Biosynthesis.

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Xylans are a diverse family of hemicellulosic polysaccharides that have a backbone structure of β-1,4 linked xylosyl residues. In some dicot species such as the model plants *Arabidopsis*, Tobacco, and Poplar, xylans from the secondary cell walls are homodisperse in backbone chain length, with the average DP found to be approximately 90 xylosyl residues. The xylans found in these model plants also possess a distinct glycoysl sequence termed "sequence 1(Seq1)" at their reducing terminus, and this structure is proposed to act as a primer for xylan biosynthesis. Mutagenic studies involving knockouts of glycosyl transferases believed to be involved in Seq1 biosynthesis resulted in severe growth phenotypes, a reduction in xylan content, reduction of detectable Seq1, as well as a loss of the homodispersity of xylan chain length. Though the majority of xylans in dicots are thought to be found within the secondary cell wall, xylans are also present in low amounts within plant primary cell walls, and recent work to characterize this primary wall xylan has found it to be structurally distinct from the xylans present within the secondary cell wall. Of the notable differences between the xylans from the two wall types include a lack of 4-O-methylation on the sidechain glucuronosyl residues, a lack of the xylan reducing end structure Seq1, as well as a loss of chain length homodispersity in primary cell walls. These findings have led to the investigation of the biochemical control of xylan synthesis in different wall types as well as the characterization of the glycosyl transferases believed responsible for synthesis of the xylan reducing end structure Seq1. In this study, family GT47 glycosyl transferases thought to play a role in xylan biosynthesis were expressed in human embryonic kidney(HEK) cells, and the activity and substrate specificity for these enzymes were examined. Structurally defined substrates of the reducing end terminus were prepared to examine the ability of these enzymes to catalyze the addition of the three different sugars and glycosyl linkages necessary to produce the reducing end structure. Furthermore, putative xylan synthases from phylogenetically diverse plant species were recombinantly expressed and assayed for activity using these substrates. The results of this study hope to gain holistic insight into the complex mechanisms governing xylan biosynthesis in various tissues and wall types and the role of the reducing end structure Seq1.

(285) The complexity of the *Ruminococcus flavefaciens* cellulosome reflects an expansion in glycan recognition

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Plant cell wall (PCW) polysaccharide degradation is an important biological and industrial process.

Non-catalytic carbohydrate binding modules (CBMs) fulfil a critical targeting function in PCW depolymerization. Defining the portfolio of CBMs, the CBMome, of a PCW degrading system is central to understanding the mechanisms by which microbes depolymerize their target substrates. Genome sequencing of *Ruminococcus flavefaciens* strain FD-1, the most abundant ruminal cellulolytic bacterium, revealed a highly elaborate assembly of scaffoldins indicating that the bacterium's cellulosome is the most intricate and versatile PCW degrading system known.

Here high throughput screening of 177 protein modules of unknown function was used to determine the complete CBMome of *R. flavefaciens*. The data identified six novel CBM families that targeted β-glucans, β-mannans and the pectic polysaccharide homogalacturonan. The crystal structures of four CBMs in conjunction with site-directed mutagenesis provides insight into the mechanism of ligand recognition. In the CBMs that recognize β-glucans and β-mannans differences in the conformation of conserved aromatic residues had a significant impact on the topology of the ligand binding cleft and thus specificity. A cluster of basic residues in CBM77 confers calcium independent recognition of homogalacturonan indicating that the carboxylates of galacturonic acid are key specificity determinants. Here we report that the extended repertoire of proteins in the cellulosome of *R. flavefaciens* contributes to an extended CBM profile that supports efficient PCW degradation.