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(P001) Structural and mechanistic studies of C-mannosyltransferase

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Tryptophan C-mannosylation is a post-translational protein modification that is essential in metazoans. It is found in secretory and membrane proteins involved in cellular communication processes such as neuroblast polarization, cytokine signaling, or spermatogenesis. The enzyme catalyzing the modification is called tryptophan C mannosyltransferase or DPY19, a member of the superfamily of C-type glycosyltransferases (GT-C). The reaction takes place in the lumen of the endoplasmic reticulum, where proteins containing the specific recognition sequon WxxW/C are modified prior to folding. A single mannose unit is transferred from dolichylphosphate-linked mannose (Dol-P-Man) to the N-terminal tryptophan of the sequon. The mechanism of substrate recognition and of the generation of a carbon-carbon glycosidic bond are unknown. We here present cryo-EM structures and in vitro functional studies of DPY19 from C. elegans. Our structures captured four distinct states of the enzyme: substrate-free, bound to acceptor peptide, bound to a synthetic donor substrate analog, and trapped in a ternary complex containing acceptor peptide and a non-reactive donor substrate analog. These structures reveal how DPY19 enzymes recognize their acceptor peptide sequon, rationalize why only unfolded proteins are processed, and suggest the basis of their specificity for Dol-P-Man over Dol-P-Glc. We found that binding of acceptor peptide induces conformational changes in the active site that contribute to the chemical activation of the donor substrate Dol-P-Man. We demonstrate that unlike other GT-Cs, DPY19 activity does not require divalent metal ions. Structure-guided mutagenesis functionally validated the roles of individual amino acids involved in substrate binding and activation. Our findings allowed us to propose a catalytic mechanism of DPY19 function and may provide the basis for future drug development.

(P002) Development of radical carbohydrate footprinting for glycan solvent accessible surface analysis

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Mass spectrometry-based protein footprinting has had considerable success in probing carbohydrate binding sites of various biomedically important proteins; however, there are few methods for quickly determining carbohydrate structures that mediate protein-carbohydrate interactions. We are developing a new approach using a mass spectrometry-based fast photochemical surface labeling of carbohydrates. Both hydroxyl radicals and trifluoromethyl radicals were investigated for their ability to probe changes in the solvent accessibility of carbohydrate in model protein-carbohydrate complexes.

Both hydroxyl and trifluoromethyl radicals were tested, both generated by sub-ms pulses of UV light with one pulse for any given sample volume. Labeled samples were digested with a protease mixture and analyzed on a Thermo Orbitrap Fusion Tribrid (Thermo Fisher Scientific) coupled with a Dionex Ultimate 3000 liquid chromatography (Thermo Fisher Scientific).

For hydroxyl radical labeling, we labeled a mixture of five trisaccharides (NAG3, and four isomeric trihexoses) with hydroxyl radicals either alone or in the presence of one of four proteins: lysozyme (which binds only NAG3 at the reducing end), G. simplicifolia (Gs) lectin (which binds only NAG3 at the non-reducing end), ubiquitin and myoglobin (which are not known to bind any of the trisaccharides). LC-MS/MS indicated labeling was specific, converting the reducing end aldehyde to a carboxylic acid. We calculated the oxidation of each trisaccharide in the presence and absence of either binding or non-binding proteins. As expected, there was no significant change in the total oxidation of NAG3 when mixed with two non-binding proteins. Similarly, there was no significant change in the oxidation of the four non-binding trihexoses when mixed with any of our test proteins. The oxidation of NAG3 was significantly decreased ($p \leq 0.05$) in the presence of two NAG3-binding proteins, lysozyme and Gs lectin. These results indicate that the binding of the glycan to its interacting protein significantly decreases the reactivity of the bound carbohydrate to hydroxyl radicals. Moreover, the reduction in labeling was significantly greater ($p \leq 0.05$) for lysozyme than Gs lectin, indicating that the hydroxyl radical footprint is sensitive to the specific regions of the carbohydrate mediating the interaction.

Studies are underway examining the utility of trifluoromethyl radicals for footprinting of carbohydrates. Preliminary results indicate that, unlike hydroxyl radicals that labeled specifically at the reducing end, trifluoromethyl radicals label the carbohydrate at multiple sites, spread among the monosaccharide units. Experiments are underway to test the ability of trifluoromethyl radicals

to successfully differentiate between binding and non-binding trisaccharides in a mixture, as well as to structurally determine which portion of the carbohydrate is mediating the interaction.

(P003) GlycoGrip: A Glycocalyx-Inspired Lateral Flow Strip-based Assay Designed to Detect Betacoronaviruses

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Inspired by the role of cell-surface glycoproteins as co-receptors for various pathogens, we report the development of *GlycoGrip*: a glycopolymer-based sandwich-type lateral flow assay for detecting SARS-CoV-2 and its rapidly emerging variants. *GlycoGrip* utilizes glycopolymers for primary capture, and anti-spike antibodies labeled with gold nanoparticles for signal-generating detection. A lock-step integration between experiments and computation has enabled efficient optimization of *GlycoGrip* test strips which can selectively, sensitively, and rapidly detect SARS-CoV-2 and its variants in biofluids. Employing the power of the glycocalyx in a diagnostic assay has distinct advantages over conventional immunoassays as glycopolymers can bind to antigens in a multivalent capacity, and are highly adaptable for mutated strains, as demonstrated by our results. As new variants of SARS-CoV-2 are identified, *GlycoGrip* will serve as a highly reconfigurable biosensor for their detection. Additionally, via extensive ensemble-based docking simulations to incorporate protein and glycan motions, we have elucidated important clues as to how heparan sulfate and other glycocalyx components bind the spike glycoprotein during the SARS-CoV-2 host-cell invasion and utilize these spike-glycocalyx interactions to optimize *GlycoGrip*'s sensing technology. *GlycoGrip* is a promising, generalizable alternative to costly, labor-intensive RT-PCR, and we envision it will be broadly useful, including for rural or low-income populations that are historically undertested and underreported in infection statistics.

(P004) Glycans as central regulators of the immunological pathways at the frontiers of microbial infections, chronic inflammation and autoimmunity.

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The immune system is coordinated by an intricate series of stimulatory and inhibitory circuits that regulate the host response to microbial challenges as well as to inflammation and autoreactive states. Disruption of these safeguard mechanisms can lead to unpredictable autoimmune pathology and exacerbated inflammation, whereas deficiency of immune-stimulatory pathways may orchestrate immunosuppressive networks that promote malignant transformation and perpetuation of chronic infections (*Pinho & Reis, Nature Rev Cancer 2015*). Glycans have recently emerged as essential components of homeostatic circuits, acting as fundamental fine-tuners of immunological responses and thus novel molecular targets for manipulation of immune activation or tolerance (*Alves,. . . ,Pinho FEBS Letter 2022; Alves,. . . , Pinho Arthritis and Rheumatol 2021*).

Glycans are present in essentially all cellular surfaces, being important regulators of the immune system. In fact, T-cells contain a dense coat of glycans (glycocalyx) that tightly regulate its activity and function *(Dias,. . . Pinho. PNAS 2018; Pereira,. . . ,Pinho Frontiers Immunol 2018)*. T-cell development ensures the formation of diverse repertoires of T-cell receptors, that recognize a variety of antigens throughout life. However, how glycans regulate thymic lymphocyte development, and how thymic T-cell glycome defines susceptibility to disease, such as inflammation and autoimmunity remain unclear.

In this study, we discovered stage-specific glycome composition along T-cell development in human and murine thymocyte. After perturbing the branching *N*-glycosylation profile of thymic T cells using specific glycoengineered mice, displaying absence of complex branched *N*-glycans and the consequent expression of mannosylated structures at DN2 stages ($Rag1^{Cre}Mgat1^{fl/fl}$), we showed remarkable defects in key developmental checkpoints, including ß-selection, regulatory T-cell generation and $\gamma\delta$T-cell lineage choice, that was associated with increased susceptibility to colon and kidney inflammation, as well as to infection. These mice showed an interesting infiltration of colon and kidney tissues with $\gamma\delta$T-cells that appears to drive the increase susceptibility to inflammation and autoimmunity. Here, we revealed that mannosylated thymocytes render a dysregulation in T-cell development, associated with inflammation susceptibility. We further demonstrated that a single *N*-glycan antenna (modelled in $Rag1^{Cre}Mgat2^{fl/fl}$) is the *sine-qua-non* condition to ensure proper T cell developmental processes.

Altogether we disclose the relevance of glycans in imprinting, early in T cell development either the pro-inflammatory or the anti-inflammatory properties of T cells and thus the susceptibility to inflammation and autoimmunity.

(P005) An atlas of the human O-Man glycoproteome reveals domain-specific modifications and substrate specificities of human mannosyltransferases

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Protein O-mannosylation (O-Man) is an evolutionary conserved post-translational modification with key functions during metazoan development. The complex O-Man glycans of α-dystroglycan (α-DG), which are initiated by the enzymes POMT1/2, are directly responsible for interactions between α-DG and extracellular matrix proteins. Additionally, biosynthetic defects in α-DG O-Man underlie multiple forms of muscular dystrophies and developmental disorders.

More recently, two new biosynthetic pathways responsible for O-Man initiation in metazoans have been described. The *TMTC1-4* family of genes encodes for O-Man glycosyltransferases that selectively modify extracellular cadherin (EC) domains found among members of the cadherin superfamily. Conversely, TMEM260 is responsible for O-Man on immunoglobulin, plexin and transcription factor (IPT) domains, which are common among plexin receptors, cMET and RON. Thus, metazoans have evolved unique O-Man glycosylation capacities that, in contrast to POMT1/2 enzymes, target folded protein domains. Additionally, mutations in TMTC1-4 or TMEM260 cause neurodevelopmental disorders, including brain malformations, structural heart defects and/or renal anomalies, indicating that O-Man localized to specific protein folds fulfills important functions during development.

The EC- and IPT-domain substrates of TMTC1-4 and TMEM260, respectively, share structural features that are common to immunoglobulin (Ig) protein domains. We hypothesized that other subclasses of Ig domains may serve as substrates for TMTC1-4 or TMEM260. To test this hypothesis, we designed a strategy for targeted membrane glycoproteomics that allows sensitive identification of O-Man glycosylations, and established an atlas of human O-Man glycoproteins expressed across different cell lines. A library of genetically engineered human cell lines with individual, combinatorial and complete deconstruction of O-Man biosynthetic pathways was used for dissection of POMT1/2, TMTC1-4 and TMEM260 substrate specificities.

Our results demonstrate the presence of O-Man glycosylation on different types of protein folds, including Ig-like C-/V-type and fibronectin domains. Moreover, our study identifies members of the immunoglobulin superfamily of cell adhesion molecules (IgSF CAMs), integrin α- and β-members and β-dystroglycan (β-DG) as O-Man glycoproteins, which adds knowledge on the emerging concept of domain-specific O-Man glycosylation. These findings open for new hypotheses and molecular mechanisms for O-Man in development and congenital disorders of glycosylation.

(P006) Mass Spectrometry-based High-throughput Profiling of Porcine Notochordal-Cell Matrix N-glycans Using GlycanExplorer™ Software

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Introduction. *N*-glycans play key roles in important biological processes such as protein folding and trafficking, cell-cell interactions and signal transduction [1]. This study analyses a potential substrate for regenerative therapies, notochordal cell derived matrix (NCM) from Porcine nucleus pulposus [2]. It is being studied due to its biological properties that promote tissue regeneration [3]. Many methods of released *N*-glycan characterization have been described, however, determination of individual glycan species and relative abundance remains an arduous process [4]. There is a need for advanced analytical tools to improve *N*-glycan characterisation in complex biological samples. In this experiment, GlycanExplorer software is assessed as a profiling tool in an automated method and compared against a standard method of manual assignment using mass spectrometry generated data.

Methods. *N*-glycans from Porcine NCM were released from glycoproteins using PNGaseF digestion and were labelled with 2-aminobenzamide (2-AB). The 2-AB labelled glycan samples were cleaned up prior to separation by a Vanquish UHPLC with FLD (Thermo Scientific) using a BEH Glycan column (1.7 μm particles in 2.1x150 mm, Waters) coupled to a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer. For structural elucidation, LC-MS^2 experiments were

conducted in negative ion mode. Expecting large heterogeneous glycan structures, MS/MS data was acquired using normalized stepped collision energy (NCE) with energies 30, 42 and 72. GlycanExplorer™ v. 1.0 was used to perform LC-MS peak processing and subsequent MS/MS database search.

Preliminary Data. This work demonstrates a comprehensive high-throughput glycan characterisation workflow in GlycanExplorer by profiling the *N*-glycome of porcine NCM. NCM has been previously characterised to contain 346 unique *N*-glycans. Using GlycanExplorer we created a custom *in-silico* fragment database of 300 glycan structures (sulfated (S) and acetylated (Ac) modifications not yet supported). The tandem mass spectra were matched against the *in-silico* fragments with deprotonated ion species $[M-H]^-$, $[M-2H]^{2-}$, $[M-3H]^{3-}$, and $[M-4H]^{4-}$.

GlycanExplorer software reads Orbitrap-derived raw data file formats, allows one to assign sample-replicate information, select the target database(s), and create an optimized processing method for high throughput identification and quantitation. High throughput search methods include both MS1-based and MS/MS based approaches for confident identification. Exportable reports containing detailed information on glycosylation profile of the sample - the glycan compositions, structures, along with annotated mass spectra - are generated. Further, reports containing relative amounts of each glycans were generated to estimate sample-specific frequency and total ion abundances of individual glycan structures. Visual reports of abundances of complex, oligomannose, hybrid *N*-glycans; acidic and neutral *N*-glycans were also generated. Thus far, the software annotated 126/300 glycan structures (42% of previously characterised structures) using the MS1-based search and 87/300 glycan structures (29%) using the MS/MS *in-silico* fragment matching approach. The software identified 40% of all complex *N*-glycans (115/286), 100% of oligomannose structures (5/5), and 75% of hybrid *N*-glycans (6/8). Current limitations prevent glycans with sulfated (S), acetylated (Ac) substituents from inclusion in the database and hence are excluded. The lowest abundant glycan identified was core fucosylated tetraantennary trigalactosylated trisialylated glycan with polylactosamine extension (FA4G3**GlcNAc1Gal1**S3) with a relative intensity of 0.0036% and the glycan M6 was the most abundant with a relative intensity of 21.75%. This automated workflow detects many abundant glycans in the NCM, however, further optimisation and validation against the established workflows are required.

References

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(P007) Role of proteoglycans in determining muscle stem cell fate during pregnancy

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Pelvic floor disorders (PFDs) are highly prevalent and costly conditions that impact quality of life of millions of women. Maternal injury to the pelvic floor muscles (PFMs) is a key risk factor for subsequent PFDs. During childbirth, PFMs elongate beyond skeletal muscle's physiological limit. Thus, injury should occur in most, if not all vaginal deliveries; however, many women do not exhibit PFM injuries. Using the validated rat model, we have shown that during pregnancy PFMs undergo longitudinal muscle growth through sarcomerogenesis. This protective adaptation enables PFMs to withstand large deformations with decreased susceptibility to mechanical birth injury. Muscle stem cells (MuSCs) are responsible for muscle adaptations under variable physiological conditions. To exert their function, MuSCs activate, proliferate, and differentiate. Regulation of MuSC fate decision depends on a complex network of niche and systemic factors. To understand potential mechanisms that govern pregnancy-induced protective PFM adaptations, we aimed to identify factors that induce antepartum MuSC activation.

We identified significant increase in PFM cell number *in vivo* in mid- (MP) compared to non- (NP) and late-pregnant (LP) animals, using immunofluorescent staining of Pax7, a pathognomonic MuSC transcription factor. EdU injections 24 hours before tissue harvest revealed increased proliferation of MP cells. In addition, myogenin, a marker of differentiated cells, was increased in MP PFMs. Together, these results suggest that MuSC activation and progression through myogenic lineage is significantly greater in MP compared to NP and LP states. To determine putative microenvironmental and systemic factors that lead to the MP MuSC phenotype, we analyzed PFM extracellular matrix (ECM) composition using liquid chromatography–tandem mass spectrometry (LC–MS/MS) and systemic hormonal levels using ELISA during pregnancy.

ECM analysis revealed significant increase in small leucine-rich proteoglycans, specifically fibromodulin, decorin, and lumican, in MP PFMs. Previous studies showed that the increase in these proteins induces MuSC differentiation through sequestration of inhibitory factors. Serum analysis showed that the low levels of progesterone (Pr) and estrogen (E) observed in NP serum increase significantly in MP and LP animals and in LP rats, respectively. It has been previously shown that high Pr levels induce increased decorin expression, while high E levels decrease fibromodulin, decorin, and lumican expression.

Our results, combined with the existing literature, lead us to hypothesize that high serum Pr in MP animals induces proteoglycans expression, in turn, increasing MuSCs differentiation, while high E levels in LP rats block this effect, allowing MuSCs to go back to quiescence. We will explicitly test this hypothesis in our ongoing studies to determine the mechanism of MuSC fate changes during pregnancy that ultimately lead to protective PFM adaptations.

(P008) The structure of EXTL3 provides insight into bi-domain GTs, the GT47 family, and the different roles of exostosins in heparan sulphate synthesis

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Glycosyltransferases (GTs) from family GT47 catalyse a remarkably wide range of glycosylations in plants and animals. In animals, GT47-family enzymes called exostosins are involved in polymerising heparan sulphate (HS), an essential extracellular carbohydrate. Like chondroitin sulphate (CS) and matriglycan, HS is first made in the Golgi as a polymer of two alternating monosaccharides—GlcA and GlcNAc in the case of HS. Most exostosins have two GT domains (GT47 and GT64), which are thought to catalyse the required GlcAT and GlcNAcT reactions, respectively. Interestingly, similar tandem-GT structures are also predicted in enzymes polymerising CS (ChSy; GT31–GT7) and matriglycan (LARGE; GT8–GT49), suggesting that natural selection consistently favours GT–GT domain fusion in enzymes making alternating polysaccharides. However, the lack of any high-resolution structure obstructs insight into the mechanistic benefit conferred by such arrangements. Moreover, a complete lack of relevant structures obscures the molecular basis for the diversity in GT47 activities. To help address these issues, we set out to investigate the structure and function of exostosin-like 3 (EXTL3), a bi-domain exostosin that initiates HS synthesis by adding the first GlcNAc residue. Interestingly, in contrast to its close homologue exostosin 1 (EXT1), EXTL3 is reported to lack GlcAT activity. Building on previous work, we purified a secretory version of EXTL3 and developed a polysaccharide analysis by carbohydrate electrophoresis (PACE)-based assay to confirm its activity. Using cryo-EM, we then solved high-resolution structures for the protein in both the absence and presence of UDP. The structures revealed that, although EXTL3's GT47 domain adopts a GT-B fold complete with a conserved acidic residue (that likely binds ribose in other GT47 members), a number of EXTL3-specific structural features prevent nucleotides and nucleotide sugars from binding in the GT47 active site—explaining previous biochemical observations. Therefore, all EXTL3 activity must originate from the GT64 domain, to which UDP was clearly bound. Our phylogenetic analysis also highlighted the existence of a previously unclassified group of GT47 sequences ('GT47-G') in non-angiosperm plants that exhibits high similarity with the animal sequences. Finally, we inferred from the overall architecture of the EXTL3 structure and an EXT1/2 AlphaFold model that HS backbone polymerisation is almost certainly distributive in mechanism. Hence, GT–GT fusion may comprise a simple method to increase local acceptor concentration in the Golgi.

(P009) Transcriptomic analyses unravel differential expression of genes involved in the N-glycosylation pathway of Phaeodactylum tricornutum ecotypes

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Most of the biologics are glycoproteins. It is well-established that *N*-glycans harboured by proteins are involved in the protein half-life, bioactivity and immunogenicity. Currently, most of the biologics are produced in mammalian cells. However, microalgae emerged as a cheaper alternative biofactory. Among them, the diatom *Phaeodactylum tricornutum* benefits from numerous advantages and has been successfully used to produce biologics such as SARS-COV2 RBD and functional monoclonal antibodies (mAbs). These mAbs have been demonstrated to be glycosylated with oligomannosides that are similar to the mammalian ones and that result from processing steps occurring in the ER and the early Golgi apparatus. Surprisingly, these oligomannosides

represent the major *N*-glycans population even if the diatom possesses glycoenzymes potentially involved in the biosynthesis of complex-type *N*-glycans in the Golgi apparatus. Therefore, it is essential to characterize the regulation of the *P. tricornutum* protein *N*-glycosylation pathway as well as the expression level of genes involved in the *N*-glycosylation of proteins. In the present work, we performed RNA-Seq analyses on different ecotypes of *P. tricornutum* and decode the differential expression of genes involved in the protein *N*-glycosylation pathway. This work was financially supported by the French government through the ANR agency under the action of the Grand Défi Bioproduction and Biologics (ANR-21-F2II-0005).

(P010) 1,3-Pr_2-6-OTs GlcNAlk as bioorthogonal precursor for specific labeling of *N*-glycan hybrid structure

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N-linked glycosylation is one of the most important, chemically complex, and ubiquitous post translational modifications in all eukaryotes. There are three types of *N*-glycan structures, high mannose, hybrid, and complex structure of which the last two types of *N*-glycans (hybrid and complex) are generated by trimming of high mannose structure by glucosidase and mannosidase enzymes. To date, there is no definitive way to distinguish among them. Here we describe synthesis of 1,3-Pr_2-6-OTs GlcNAlk as next generation metabolic chemical reporter (MCR) for the specific labeling of hybrid *N*-glycan structure. We used a series of *N*-glycan trimming pathway inhibitory experiments, along with MGAT1 siRNA knockdown to establish that this bioorthogonal sugar is specific and enzymatically incorporated into hybrid *N*-glycans. Using this MCR as the detection tool, we detect previously underappreciated nuclear hybrid *N*-glycosylation and define the nucleolar protein fibrillarin as modified with this MCR.

(P011) Immunoglobulin N-glycosylation Discriminates Acute Lyme disease from Endemic Healthy Controls and Mimic Diseases – A Novel Diagnostic and Prognostic

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Current diagnostics for acute Lyme disease (LD) suffer from a sensitivity of 46% leading to a high false-negative rate. As a result, patients with undetected cases of Lyme disease delay antibiotic treatment and risk bacterial dissemination to their brain, joints, and heart. We present evidence that patients' global immunoglobulin G and M (IgG, IgM) glycosylation is specifically altered during acute LD. This approach uses UPLC-FLR-ESI-MS to quantitate and identify N-glycans from purified glycoproteins. While inflammatory diseases canonically induce global IgG with increased F(6)A2G0 N-glycan content, we detect a marked decrease of F(6)A2G0 during acute Lyme disease – below that of healthy control levels. Agalactosylated species such as F(6)A2G0 are known to promote FcγR IIIA signaling on circulating lymphocytes to promote inflammation. Yet during acute Lyme disease, the IgG Fc fragment increases the galactose and sialic acid content. This finding suggests a novel immunomodulation induced by acute *Borrelia burgdorferi* infection that permits evasion of adaptive immunity. Moreover, in IgM, we have detected acute LD-specific alterations of mannosylated and complex-type N-glycan abundance. Little research has been conducted on the impact of altered glycosylation on IgM N-glycans, but reports suggest that rates of complement deposition and T-cell activation are at least in part controlled by the glycosylation profile of IgM. Recognizing the clinical need for an accurate test for acute Lyme disease, we analyzed the global IgG and IgM N-glycome from 18 acute Lyme disease patients compared to 18 endemic healthy controls. We determined this approach is 72% sensitive and 100% specific. Next, we analyzed the CDC Lyme disease Panel 1 and determined that, unlike current diagnostics, our approach detects N-glycomic changes before seroconversion is detected on western blots or ELISA assays. The glycan-based assay was further examined by testing seronegative early acute LD, convalescent acute LD, late-stage untreated LD, endemic healthy controls, non-endemic healthy controls; and LD-mimic diseases: Rheumatoid arthritis, Fibromyalgia, Mononucleosis, Multiple Sclerosis, and Syphilis. We determined that the global IgG and IgM N-glycome correctly identified only acute LD patients because the lack of the canonical increase of F(6)A2G0 discriminated acute Lyme disease from all other LD-mimic diseases, healthy controls, and late-stage Lyme. Lastly, when patients donated serum at a convalescent time point, we identified significantly increased bisecting N-glycans suggesting an increased immunologic response following antibiotic treatment for acute LD. Taken together, glycomic biomarkers for acute Lyme disease hold immense potential to be developed into a novel and much-needed diagnostic and prognostic.

(P012) Spatiotemporal biosynthesis of paucimannosidic proteins via a noncanonical truncation pathway in human neutrophils

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We recently discovered that human neutrophils express immunomodulatory glycoproteins decorated with unusual and highly truncated paucimannosidic *N*-glycans ($Man_{1\text{-}3}GlcNAc_2Fuc_{0\text{-}1}$), but their biosynthesis remains unexplored. Guided by the well-characterised truncation pathway in invertebrates and plants in which the *N*-acetyl-β-D-hexosaminidase (Hex) isoenzymes are known to catalyse the formation of paucimannosidic proteins (PMPs), we here set out to test if homologous human Hex isoenzymes encoded by *HEXA* and *HEXB* drive a similar truncation pathway in human neutrophils. Firstly, we performed quantitative glycomics and glycoproteomics of several CRISPR-Cas9-edited Hex-disrupted neutrophil-like HL-60 mutants (*HEXA-KO* and *HEXB-KO*) and unedited control cell lines. Hex disruption was validated using next-generation sequencing, ELISA, proteomics and Hex activity assays. Excitingly, all Hex-disrupted mutants displayed significantly reduced levels of paucimannosylation, particularly of $Man_{2\text{-}3}GlcNAc_2Fuc_1$, relative to unedited HL-60 suggesting that both *HEXA* and *HEXB* contribute to PMP formation via a previously unexplored truncation pathway in human neutrophils. Quantitative *N*-glycomics indeed demonstrated reduced utilisation of a putative noncanonical truncation pathway in favour of the canonical elongation pathway in all Hex-disrupted HL-60 mutants relative to unedited controls. Quantitative glycoproteomics confirmed a truncation-to-elongation switch in all Hex-deficient HL-60 mutants and showed a pronounced switch for *N*-glycoproteins co-trafficking with Hex to the azurophilic granules of neutrophils including myeloperoxidase. To support the Hex-paucimannose link, we then documented that neutrophils from an early-onset Sandhoff disease patient ($HEXB^{-/-}$) displayed dramatically reduced paucimannosylation relative to neutrophils from an age-matched unaffected donor. Further, re-interrogation of publicly available proteomics and transcriptomics data from bone marrow-derived immature granulocytes suggested a maturation-dependent utilisation of the truncation pathway in primary neutrophils. Glycoprotein truncation was favoured in early neutrophil maturation (promyelocytes), and gradually reduced during granulopoiesis in favour of glycoprotein elongation. The temporal truncation-to-elongation switch was supported by a dynamic glyco-gene expression during granulopoiesis. *HEXA*, *HEXB* and genes encoding α-/β-mannosidases (*MAN2B1*, *MANBA*) were predominantly expressed in promyelocytes while the expression of the elongation glyco-enzymes (*B4GALT3/4*, *ST3GAL2*, *ST6GAL1*) dominated in late-stage neutrophil maturation. Finally, quantitative glycoproteomics on isolated neutrophil granules showed that PMPs were indeed enriched glycosignatures in the early-formed azurophilic granules relative to other granule populations. We conclude that human Hex mediates spatiotemporal formation of PMPs via a dynamically utilised noncanonical truncation pathway in maturing human neutrophils.

(P013) The Cup of Life is Not So Shallow: Milky Secrets

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Milk oligosaccharides (MOs) are essential for infant immunity, nutrition, and development. In many instances, clear sequence-to-function relationships have linked milk glycan motifs to these roles. Yet, our understanding of milk glycan diversity and evolution is strongly biased toward human and cow MOs. Here, we engaged in detailed structural glycomics for eight uncharacterized mammalian species, reporting the discovery of new glycans, motifs, and even monosaccharides in milk. Overall, we discovered more than 50 novel MO structures and increased the total number of milk glycan reports by more than 10%, leading to a re-evaluation of the true diversity of MOs in Mammalia. We further developed and applied a comprehensive computational workflow, fully integrated into the glycowork platform, to automatically generate and analyze biosynthetic networks of milk oligosaccharides. On a curated dataset of milk glycans from over 100 mammalian species, we reveal (i) potential experimental biases, (ii) biosynthetic restrictions, such as reaction path dependence, and (iii) conserved biosynthetic modules. Using machine learning and network analysis, we identify characteristic sequence relationships and evolutionary gains/losses of motifs, MOs, and biosynthetic modules in mammalian groups. These resources and analyses will advance our understanding of the biosynthesis of complex carbohydrates and the evolution of breast milk as an important nutrient.

(P014) Specific N-glycans regulate the function of an extracellular adhesion complex during somatosensory dendrite patterning

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N-glycans are molecularly diverse sugars borne by over 70% of proteins transiting the secretory pathway and have been implicated in protein folding, stability, and localization. Mutations in genes important for *N*-glycosylation result in congenital disorders of glycosylation that are often associated with intellectual disability. Here, we show that structurally distinct *N*-glycans regulate an extracellular protein complex involved in the patterning of somatosensory dendrites in *Caenorhabditis elegans*. Specifically, *aman-2/Golgi alpha-mannosidase II*, a conserved key enzyme in the biosynthesis of specific *N*-glycans, regulates the activity of the Menorin adhesion complex without obviously affecting protein stability and localization of its components. AMAN-2 functions cell-autonomously in somatosensory dendrites to allow decoration of the DMA-1/LRR-TM neuronal leucine rich transmembrane receptor with the correct set of high-mannose/hybrid/paucimannose *N*-glycans. Moreover, distinct types of *N*-glycans on specific *N*-glycosylation sites regulate DMA-1/LRR-TM receptor function, which, together with three other extracellular proteins, forms the Menorin adhesion complex. We propose that specific *N*-glycan structures regulate dendrite patterning by coordinating the activity of an extracellular adhesion complex suggesting that the molecular diversity of *N*-glycans can contribute to developmental specificity during development of the nervous system.

(P015) Sequential *in vitro* enzymatic N-glycoprotein modification reveals site-specific rates of glycoenzyme processing

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N-glycosylation is an essential eukaryotic post-translational modification that affects various glycoprotein properties, including folding, solubility, protein-protein interactions, and half-life. N-glycans are processed in the secretory pathway to form varied ensembles of structures, and diversity at a single site on a glycoprotein is termed 'microheterogeneity'. To understand the factors that influence glycan microheterogeneity, we hypothesized that local steric and electrostatic factors surrounding each site influences glycan availability to enzymatic modification. We tested this hypothesis by expression of a panel of reporter N-linked glycoproteins in *MGAT1*-null HEK293 cells to produce immature $Man_5GlcNAc_2$ glycoforms (38 glycan sites total). These glycoproteins were then sequentially modified *in vitro* from high-mannose to hybrid and on to biantennary, core fucosylated, complex structures by a panel of N-glycosylation enzymes and each reaction time-course was quantified by LC-MS/MS. Substantial differences in rates of *in vitro* enzymatic modification were observed between glycan sites on the same protein and differences in modification rates varied depending on the glycoenzyme being evaluated. By comparison, proteolytic digestion of the reporters prior to N-glycan processing eliminated differences in *in vitro* enzymatic modification. Comparison of *in vitro* rates of enzymatic modification with the glycan structures found on the mature reporters expressed in wild type cells correlate well with the enzymatic bottlenecks found *in vitro*. These data suggest that higher-order local structures surrounding each glycosylation site contribute to the efficiency of modification both *in vitro* and *in vivo* to establish the spectrum of site-specific microheterogeneity found on N-linked glycoproteins.

(P016) Opposing Functions of Galectins 3 and 8 in Modulating Differentiation State at Homeostasis and in Metaplasia

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Although aberrant expression of neoglycosylation epitopes is widely recognized to occur in metaplasia and cancer where they serve as important biomarkers to (1) diagnose these tissue transformations, (2) monitor therapeutic response, and (3) evaluate for recurrence, the functional consequences of these post-translational modifications are under- and/or unstudied. In the gastrointestinal foregut, galactose containing Lewis antigens are prominent examples of such neoglycosylation epitopes in metaplasia and cancer of the esophagus, stomach, and pancreas [e.g., 3'-Sulfo-LeA/C that we have recently described in addition to others like 3'-Sialyl-LeA (CA19-9)]. To begin to learn why these glycosylation epitopes are expressed during tissue transformation, we studied the consequences of mice null for either Galectin-3 or Galectin-8, two proteins previously shown to

preferentially bind lewis antigens that have been sulfated. Lgals3 is among the top 20 most upregulated genes in gastric cancer, while Lgals8 is generally downregulated in cancer potentially suggesting distinct or opposing cellular functions. Accordingly, we observed mice null for Lgals3 are unable to efficiently secrete these sulfomucins as they downscale their cellular architecture en route to a metaplastic phenotype. In contrast, Lgals8–/– mice exhibit a significant delay in cellular maturation as judged by the absent expression of gastric intrinsic factor or sulfomucins in the zymogenic granules of the gastric chief cells until ~4 weeks of age. Thus, galectins 3 and 8 appear to have fundamentally opposite effects on cellular differentiation, with galectin 3 overexpressed in cancer driving the cells towards a dedifferentiated state, while galectin 8 whose expression is decreased in cancer favors a mature homeostatic phenotype. The molecular mechanisms (autophagy, vesicular trafficking, exocytosis) responsible for these phenotypes will be discussed. Overall, the results presented here suggest that the expression of neoglycosylation epitopes along with their receptors plays an important role in modulating cellular architecture both at homeostasis and in processes like paligenosis. As such, neoglycosylation epitopes appear to confer unique cellular plasticity to cells enabling them to undergo metaplastic and oncogenic transformation.

(P017) Deciphering O-glycoprotease substrate preferences with O-Pair Search

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O-glycoproteases are an emerging class of enzymes that selectively digest glycoproteins at positions decorated with specific O-linked glycans. O-glycoprotease substrates range from any O-glycoprotein (albeit with specific O-glycan modifications) to only glycoproteins harboring specific O-glycosylated sequence motifs, such as those found in mucin domains. Their utility for multiple glycoproteomic applications is driving the search to both discover new O-glycoproteases and to understand how structural features of characterized O-glycoproteases influence their substrate specificities. One challenge of characterizing O-glycoprotease specificity restraints is the need to characterize O-glycopeptides with site-specific analysis of O-glycosites. Here, we demonstrate how O-Pair Search, a recently developed O-glycopeptide-centric identification platform that enables rapid searches and confident O-glycosite localization, can be used to determine substrate specificities of various O-glycoproteases *de novo* from LC-MS/MS data of O-glycopeptides. Using secreted protease of C1 esterase inhibitor (StcE) from enterohemorrhagic *Escherichia coli* and O-endoprotease OgpA from *Akkermansia mucinophila*, we explore numerous settings that effect O-glycopeptide identification and show how non-specific and semi-tryptic searches of O-glycopeptide data can produce candidate cleavage motifs that can be used to define new protease cleavage settings that lower search times and improve O-glycopeptide identifications. We use this platform generate a consensus motif for the recently characterized immunomodulating metalloprotease (IMPa) from *Pseudomonas aeruginosa* and show that IMPa is a favorable O-glycoprotease for characterizing densely O-glycosylated mucin-domain glycoproteins.

(P018) *FUT*1 Knockout Leads to Reduced α(1,2)-Fucosylated Epitope Expression and Reduced Receptor Tyrosine Kinase Signaling in Head and Neck Cancer Cells

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Most head and neck squamous cell carcinomas (HNSCC) arise from the mucosal epithelium in the oral cavity, pharynx and larynx, and HNSCC risk factors include tobacco and alcohol use, and human papillomavirus (HPV) infection. Aberrant glycosylation is a hallmark of tumor cells, but the role of aberrant glycosylation has not been well defined in HNSCC. The α(1,2)-fucosyltransferase FUT1 is associated with poor prognosis, and drives self-renewal/stemness, migration, invasion, adhesion, and drug resistance in multiple cancer types. To understand the potential contribution(s) of FUT1 to aggressive tumor characteristics in HNSCC, we have employed tissue immunohistochemistry of human HNSCC samples, CRISPR-editing of established HNSCC cell lines, flow cytometry, transcriptomics, and immunoblotting of HNSCC cell line lysates. CRISPR-mediated knockout of *FUT*1 dramatically reduces the surface expression of α(1,2)-fucosylated epitopes. The observed reduction in α(1,2)-fucosylation broadly impacts the phosphorylation of multiple receptor tyrosine kinases (RTKs) and downstream signaling targets, including but not limited to, EGFR-signaling pathways. Protease digestion studies indicate that a sizeable proportion α(1,2)-fucosylated epitopes are protease-resistant. Ongoing mucinomic and *N*-glycoproteomic studies seek to define these FUT1 glycoprotein substrates. Given that surface receptors that govern critical cellular signaling cascades are highly modified by *N*- and *O*-linked glycans, we propose that α(1,2)-fucosylated glycans alter the activity of these molecules, leading to the dysregulation of cell signaling in HNSCC, thereby thus serving as critical drivers of HNSCC carcinogenesis.

(P019) Peptide Substrate Charge Distributions: A New Hallmark Governing GalNAc-T Specificity

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Mucin type O-glycosylation is initiated by a family of 20 GalNAc-Ts. These enzymes define sites of O-glycosylation by transferring of an α-GalNAc from a UDP-GalNAc donor to the hydroxyl groups of Ser and Thr residues. Mutations and differential expression of several GalNAc-Ts have been linked to/associated with many disease states including several types of cancers. However, the mechanism by which these isozymes choose their targets is not fully understood. Our lab has shown that the GalNAc-Ts possess unique specificities for: 1) Thr over Ser residues, 2) peptide sequence, and 3) prior peptide glycosylation (1). Recent studies on GalNAc-T3 and the fly PGANT9A/B splice variants have shown that flanking charged residues (≥-/+4 residues from the site of glycosylation) may alter the rates of glycosylation (2,3). To systematically study the roles of flanking charge on GalNAc-T activity, we characterized a series of charged peptide substrates containing all combinations of positive, negative, and neutral charged residues flanking a common glycosylation site (YAV**T**PGP) against 12 of the 20 GalNAc-Ts. We found isozyme preferences for positive, negative, or unique combinations of positive/negative charge flanking the site of glycosylation that correlated to their electrostatic surface charge distribution. Interestingly, each isozyme had a unique surface charge distribution that differed from each other. Kinetic assays were performed on a subset of isozymes against their least active and most active substrates. Although the central binding sequence remained the same, the Vmax, kcat, and Km values widely differed between the substrates revealing the role of remote substrate charge recognition. As expected, reactions under elevated ionic strength, designed to disrupt charge-charge interactions, leveled out the activities of the charged substrates, thus confirming the role of surface electrostatics in transferase selectivity. Molecular dynamic simulations were conducted against several GalNAc-Ts bound to their least active and most active charged substrates. We found the N and C termini of the most active substrates interacted with the enzyme surface while the least active substrates extended away from the surface, consistent with the presence of charge-charge interactions. Overall, we have shown flanking substrate charge as another mechanism for which these isozymes choose their substrates and furthermore that these isozymes utilize a much larger surface for recognizing substrate sequences than what has been previously thought. Together, this information will better our understanding GalNAc-T specificity, their roles in disease such as cancer, and for improving isoform specific predictions of O-glycosylation.

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(2) de las Rivas et al, *Nat. Chem. Biol*, 2020, 16:351-360.
(3) May et al, *J. Biol. Chem.* 295:12525-12536 (2020).

(P020) Enabling Large-scale Glycoproteomics Data Analysis with the MSFragger Glyco Software Suite

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Analysis of glycopeptides by tandem mass spectrometry can provide site-specific annotation of glycans at a proteome-wide scale, presenting enormous potential for discoveries in glycobiology and disease. Confidently annotating the complex mass spectra produced by glycopeptides has presented a major obstacle to the realization of large-scale glycoproteomics, however. The simultaneous fragmentation of both peptide and glycan in collisional activation tandem MS experiments fundamentally impedes searching by traditional (proteomics-style) database search methods, but provides an opportunity for complete characterization of glycopeptides using advanced search methods that take advantage of the additional information provided by these fragments.

Here, we present the MSFragger Glyco search and accompanying tools in the FragPipe software suite and graphical interface. We provide a fast and sensitive peptide-first search method for glycopeptides, separate peptide and glycan scoring and false discovery rate (FDR) estimation, spectrum visualization for glycopeptides directly from the FragPipe user interface, and quantitation tools for both labeled and label-free experimental workflows. The peptide-first search method provides flexible matching of glycopeptides even if spectra do not contain a clear series of Y-ions, improving sensitivity compared to glycan-first searches, especially for O-glycopeptides. Glycan-specific annotation and scoring takes place after the initial search has identified a peptide sequence and total glycan mass, using glycan fragment ions and the observed glycan mass to determine the most likely glycan composition for the spectrum. We have recently improved this method by supplementing the original empirical scoring method for glycan fragment ions with glycan-specific fragment propensity information learned from high-confidence annotations in the dataset being processed, increasing the robustness of the method to varied fragmentation conditions. In comparison with pGlyco3 and Byonic, we show this improved method is able to annotate many more glycopeptide spectra while also providing

better accuracy of assigned glycans in an entrapment search of a combined yeast and mouse glycan database against yeast glycopeptide spectra.

FragPipe provides shareable workflows for complete processing of glycoproteomics data using these methods with a single click. For quantitative experiments, this can include generation of multi-level reports summarizing glycopeptide abundances to individual glycan-glycosite combinations, overall glycosites, glycopeptides, glycoproteins, or genes, with the aim of supporting a wide range of glycoproteomics analyses and applications. Batch processing and high-performance computing support are also now readily available using the new GUI-free mode of FragPipe to support large-scale glycoproteomics, providing a powerful, convenient, and continuously growing software suite for glycoproteomics.

(P021) IgA nephropathy: an autoimmune kidney disease with pathogenic circulatory immune complexes consisting of polymeric IgA1 with galactose-deficient and minimally sialylated *O*-glycans, IgG, and complement C3

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IgA nephropathy (IgAN) is an autoimmune kidney disease wherein pathogenic immune complexes (IC) in the circulation contain IgA1 with some hinge-region *O*-glycans deficient in galactose (galactose-deficient IgA1; Gd-IgA1) bound by IgG autoantibodies. Some of these IC deposit in the glomeruli, activate mesangial cells, and induce kidney injury. These glomerular immunodeposits are enriched for galactose-deficient glycoforms of IgA1 and the corresponding IgG autoantibodies. However, the composition of circulating IC in IgAN is not well understood.

Using lectin-affinity and size-exclusion chromatography (SEC), we isolated different molecular forms of IgA1 from sera of 20 patients with IgAN. Monomeric IgA1 (mIgA1), polymeric IgA1 (pIgA1), and IgA1 bound in IC (IgA1-IC) were analyzed for degree of galactose deficiency by a lectin binding assay performed without and with neuraminidase treatment to remove sialic acid. For assessment of biological activity, IgA1-IC were tested for proliferation-stimulating and signaling-inducing activities in cultured primary human mesangial cells. SDS-PAGE immunoblotting was used for detection of IgA, IgG, complement C3, and phosphorylated (P-) and total ERK1/2, and P- and total Akt. C3, C3b, and iC3b standards were used in SDS-PAGE under reducing conditions for comparison with the C3 forms in IC.

Distribution of the molecular forms of serum IgA1 showed mIgA1 ~90%, pIgA1 ~9%, and IgA1-IC <0.4%. The relative degree of galactose deficiency of IgA1 was highest in IgA1-IC, less in pIgA1, and least in mIgA1. IgA1 in IC had minimally sialylated *O*-glycans. The IgA1-IC had molecular mass >700 kDa. These circulatory IC induced signaling (e.g., P-ERK1/2, P-Akt) and cellular proliferation of mesangial cells. These biologically active IgA1-IC contained pIgA1, IgG, and complement C3, including C3b and iC3b.

In summary, the pathogenic circulatory IC in patients with IgAN contain IgA1 with a high degree of galactose deficiency and minimal sialylation, IgG, and complement C3, C3b, and iC3b. Future studies are needed to determine molecular details of these IC, including stoichiometry of individual components.

(P022) Structural insights into the unique substrate recognition mode of the *Toxoplasma gondii* mucin-type O-glycosyltransferase TgGalNAc-T3

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Mucin-type O-glycosylation is a post-translational modification that results in the addition of O-glycans to Ser or Thr residues on proteins and is conserved across eukaryotes, including the parasitic microorganism *Toxoplasma gondii (T. gondii)*, an obligate intracellular protozoan parasite that infects one-third of the world's population and causes toxoplasmosis. Host infection by *T. gondii* is associated with the formation of latent cysts in the central nervous system that are enveloped with a structure called the cyst wall, whose rigidity is imparted by the O-glycosylation of the mucin-like domain of the cyst wall protein CST1. *T. gondii* contains 5 members of a conserved family of enzymes termed N-acetylgalactosaminyltransferases (GalNAc-Ts), which initiate mucin-type O-glycosylation: TgGalNAc-T1, T2, T3, T4 and T5. TgGalNAcT2 is required for the initial glycosylation of the mucin-like domain of CST1, and neighboring acceptor sites are then O-glycosylated by the glycopeptide preferring isoenzymes TgGalNAcT-T1 and T3. Previous studies showed that TgGalNAc-T2 and -T3 deletion mutants produce various O-glycosylation defects on the cyst wall and reduce the infectivity of the parasite. Reduced sequence homology between *T. gondii* and metazoan GalNAc-Ts hints that an evolutionary divergence may have occurred in substrate recognition and enzyme function,

suggesting that TgGalNAc-T2 and T3 may be specifically inhibited and are thus potential drug targets for toxoplasmosis. X-ray crystal structures of TgGalNAc-T3 in complex with glycopeptides from 2.5-2.9 Å resolution show that TgGalNAc-T3 substrate specificity is dictated by a unique GalNAc binding pocket that recognizes an existing GalNAc on a substrate one amino acid N-terminal to the acceptor site. The structures also reveal a 2nd novel metal binding site that appears to help align the GalNAc binding pocket, and mutations of residues involved in both GalNAc, and 2nd metal binding reduce enzymatic activity in vitro. In the future, we aim to understand how the unique GalNAc pocket and 2nd metal site residues affect TgGalNAc-T3 function in vivo to further build a foundation for understanding and targeting mucin-type O-glycosylation in cyst wall formation and *T. gondii* pathogenesis.

(P023) Metabolic interplay between glycogen and N-linked glycans is critical for pulmonary fibrosis progression

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Spatial metabolomics is revolutionizing our understanding of the tissue microenvironment. Here, we demonstrate the application of high-dimensionality reduction and spatial clustering (HDR-SC), histopathological annotation, and histopathological prediction of matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) datasets for the comprehensive assessment of tissue metabolic heterogeneity in human formalin-fixed paraffin-embedded (FFPE) sections of lung diseases. Using this approach, we identified glycogen as a previously unknown metabolic hallmark of pulmonary fibrosis (PF). When paired with N-linked glycans, glycogen improved predictability to nearly 100% accuracy for the histopathological identification of fibrotic regions within human lung diseases. Further, a mouse model of glycogen utilization deficiency exhibited a nearly 70% reduction in fibrosis development when compared to WT animals in the bleomycin-induced PF model. This study establishes a new workflow for the analysis of MALDI-MSI complex carbohydrate datasets and identifies glycogen metabolism as a previously unknown metabolic event and a future therapeutic target for the treatment of PF.

(P024) Studying the role of α2,6-linked sialic acid in human dendritic cell biology through CRISPR-based ST6GAL1 knockout in MUTZ-3 cells

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Dendritic cells (DCs) play an essential role in the immunological network as mediators between innate and adaptive immunity. Based on studies using broader acting sialidases and metabolic inhibition of sialylation, cell surface sialylation has been suggested to play a regulatory function in DC biology including activation and maturation.[1–3] Furthermore, DC maturation is associated with reduced expression of 2,6-sialyltransferase 1 (ST6GAL1).[2,4,5] Improving the understanding of the role of glycosylation in human DC biology, requires development of suitable cell systems as well as engineering technologies to modulate specific enzymes or glycosylation pathways. The human acute myeloid leukemia cell line MUTZ-3, can be differentiated and matured in a cytokine dependent manner to fully functional human dendritic cells.[6,7] While being the currently best cell line-based DC model, MUTZ-3 is inherently hard to engineer.

In the current study we established and optimized a workflow enabling successful gene editing of MUTZ-3 using CRISPR-Cas9; besides optimization of cultivation techniques, a high knock-out efficiency and pool approaches were key to successful outcomes. Here we report the effects of CRISPR-Cas9 ST6GAL1 knockout (ST6GAL1-/-) and resulting lack of α(2,6)-linked *N*-glycan sialylation in MUTZ-3 DC biology. Sanger Sequencing in combination with ICE CRISPR Analysis Tool, as well as cell surface lectin stainings, confirmed a 90% knockout efficiency of ST6GAL1 as well as the stability of the pool and continued functional knock-out of ST6GAL1-/-.

Our data indicates that ST6GAL1 KO alone did not result in any major alterations in cellular phenotype or expression of immune related cell surface receptors in steady state. Furthermore, ST6GAL1-/- cells responded to cytokine-induced differentiation by morphological changes, as well as upregulation of the DC markers CD209, CD1a, CD80 and CD86, and downregulation of CD34, while they maintained an immature phenotype (iDC, CD83 negative). Compared to WT, ST6GAL1-/- iDCs appeared to express more co-stimulatory and antigen presenting receptors including HLA-DR, CD1a, and CD80, which is in line with previous findings. Upon activation of iDCs with a high-dose TNFα we surprisingly found, that ST6GAL1-/- exhibited fewer CD83+ cells (mature DCs) and lower upregulation of co-stimulatory molecules CD40, CD80 and CD86, indicating a lower T-cell activating potential, which is contradictory to previous data obtained using enzymatic sialic acid removal or general metabolic inhibition of sialylation.

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(P025) GlycoCAP: A cell-free GLYCOsylation platform for building Clickable Azido-sialoglycoProteins

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Lectins represent a class of promising therapeutic targets, but tools for making glycan-based therapeutics remain limited. To address this need, we have developed a cell-free glycosylation platform for building clickable azido-sialoglycoproteins (GlycoCAP). A key feature is the use of an *Escherichia coli*-based cell-free protein synthesis (CFPS) system that offers a blank slate for glycoengineering. By supplementing necessary building blocks and cofactors with glycosyltransferase-encoded plasmids, we can express a range of different bacterial glycosyltransferases to construct minimal glycan structures on a desired protein target. This system yields a highly homogeneous glycan population and allows supplementation of non-canonical sugars. As a model, we constructed a minimal trisaccharide with a terminal azido-sialic acid. Through a series of optimizations, we've achieved >60% sialylation efficiency with a non-canonical azido-sialic acid. The azido group on the sialic acid serves as a handle for click chemistry conjugation for optimizing lectin binding pocket interactions with small chemical modifications of the glycan. We anticipate that this platform will grant access to a wide range of glycan structures that can be used to therapeutically target lectins in a range of disease applications.

(P026) A novel family of sugar-specific phosphodiesterases that remove zwitterionic modifications of *N*-acetylglucosamine

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Phosphorylcholine (PC) and phosphoethanolamine (PE) are zwitterionic post-glycosylation modifications found on glycans of parasitic nematodes and other invertebrates. It is believed PC helps parasites evade host immunity. PC modifications present on N-glycans could be a potential biomarker to detect parasitic infections, which could be used to develop new diagnostic tests and novel anti-filarial drugs [1, 2]. Currently, only harsh chemical methods are known to remove PC or PE from glycans, and to our knowledge, no enzymatic analytical methods have been described to address zwitterionic glycan modifications. An enzyme capable of removing PC/PE from glycans would both improve the structural analysis of zwitterion-modified glycans and be an impactful tool in parasitology research.

In this study, we used functional metagenomic screening of a human fecal DNA fosmid library to find an enzyme that acts upon a PC modification at the 6-carbon of N-acetylglucosamine (GlcNAc), a common PC-modification site. We identified a phosphodiesterase capable of removing both PC and PE zwitterionic modifications from GlcNAc. We used ultra-performance liquid chromatography to characterize the enzyme's specificity on a variety of synthetic zwitterion modified monosaccharides and show it acts highly specifically on GlcNAc-6-PC or -PE. The phosphodiesterase is a member of the large endonuclease/exonuclease/phosphatase (EEP) superfamily where it defines a distinct subfamily of related sequences of previously unknown function, mostly from *Clostridium* bacteria species. Finally, we used matrix-assisted laser desorption/ionization mass spectrometry to analyze the enzyme's function on intact glycans from the parasitic nematode *Brugia malayi*. We found the enzyme removes PC from GlcNAc-6-PC in *N*-linked glycans and glycosphingolipid glycans, but only when the modified sugar is

in the terminal position of the oligosaccharide. This work both defines a new family of glycan-specific phosphodiesterases and yields a novel enzymatic tool for elucidation of GlcNAc-6-PC or PE in glycans.

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(P027) Fine-Tuning The Spike: Role of the Nature and Topology of the Glycan Shield in the Structure and Dynamics of SARS-COV-2

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The SARS-CoV-2 spike (S) is a type I fusion glycoprotein, responsible for initiating the infection leading to COVID19. As a feature unique of SARS-CoV-2, the thick glycan shield covering the S protein is not only essential for hiding the virus from immune detection, but may have a functional role. These newly discovered functions of the glycan shield suggest the evolution of its sites of glycosylation is potentially intertwined with the evolution of the overall protein sequence to affect optimal activity. In this work we use multi-microsecond molecular dynamics simulations with different N-glycans at key functional sites, namely N234, N165 and N343. We also assessed the effect of a change in the SARS-CoV-2 S glycan shield's topology at N370, due to the recently acquired T372A mutation. Our results indicate that the structures of the N-glycans at N234, N165 and N343 affect the stability of the active (or open) S conformation, and thus its exposure and accessibility. Furthermore, while glycosylation at N370 stabilizes the open S conformation, we find that the N370 glycan binds the closed receptor binding domain (RBD) surface, essentially tying the closed protomers together. These results suggest that the loss of the N370 glycosylation site in SARS-CoV-2 may have increased the availability of the open S form, perhaps contributing to its higher infectivity relative to CoV1 and other variants carrying the sequon. Finally, we discuss these specific changes to the topology of the SARS-CoV-2 S glycan shield through ancestral sequence reconstruction of select SARS strains and discuss how they may have evolved to affect S activity.

(P028) Therapeutic blockade of Galectin-1, but not PD-1, shapes antitumor immunity in colitis-associated colorectal cancer

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Colorectal cancer (CRC) represents the third most common malignancy and the second leading cause of cancer-related deaths worldwide. Although immune checkpoint blockade therapies have achieved long-term responses in several malignancies, in colorectal cancer (CRC), patients clinical benefit is only observed in heavily mutated tumors that are mismatch-repair–deficient or have high microsatellite instability. This limitation urges the identification of novel immune escape mechanisms and the design of additional immunotherapeutic modalities.

During the last decades, aberrant glycosylation has become an important hallmark of inflammation and tumor progression. Galectin-1 (Gal-1), an endogenous glycan-binding protein, induces tolerogenic programs and contributes to tumor cell evasion of immune responses. We show that Galectin-1 (Gal-1) confers immune privilege to CRC by increasing the frequency of $CD8^+$ regulatory T cells (Tregs) and accentuating their immunosuppressive activity in experimental models. Mice lacking Gal-1 ($Lgals1^{-/-}$) developed a lower number of tumors and showed a decreased frequency of a particular population of $CD8^+CD122^+PD\text{-}1^+$ Tregs in the azoxymethane-dextran sodium sulfate model of colitis-associated CRC (CACRC). Moreover, silencing of tumor-derived Gal-1 in the syngeneic CT26 CRC model resulted in reduced number and attenuated immunosuppressive capacity of $CD8^+$ Tregs, leading to slower tumor growth.

Furthermore, we performed transcriptomics analysis in WT and Gal1 KO mice treated with AOM-DSS. Differential expression analysis and functional enrichment showed downregulation of angiogenesis-associated pathways. Moreover, perturbation-response analysis of 14 key cancer-associated processes showed that Gal1 KO mice presented lower scores for VEGF, Hypoxia,

TNF-α and NF-κB. Accordingly, analysis of CRC patient datasets revealed a "poor prognosis signature" characterized by high Gal-1 expression and elevated $CD8^+$ Treg score.

To validate Gal-1 as a therapeutic target in CRC, we designed and biochemically characterized a new neutralizing anti-Gal-1 monoclonal antibody (mAb-3). In contrast, to anti-PD-1 mAb, which showed no beneficial effect in the AOM/DSS model, therapeutic administration of anti-Gal1 mAb-3 in the experimental murine model of CCRAC resulted in a significant decrease in the total number of tumors and the total tumor volume. Thus, targeting Gal-1/glycan interactions may represent a promising immunotherapeutic modality for treating CRC.

(P029) MALDI Imaging Mass Spectrometry Reveals N-glycan and ECM Peptide Signatures of Renal Cell Carcinoma Immunotherapy Response

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Kidney cancer survival rates are typically poor, especially so for those diagnosed with clear cell renal cell carcinoma (ccRCC). Treatment with immunotherapeutic checkpoint inhibitors enhances survival for some ccRCC patients, yet development of therapeutic resistance and overall non-responsiveness remains common. Checkpoint inhibitors engage their targets extracellularly, thus characterizing extracellular matrix (ECM) and cell-surface components which may typify therapeutic response is of significant clinical interest. Identified ECM and glycan signatures which differentiate ccRCC responders may provide insight into resistance mechanisms or allow prognostic stratification of patients. For this purpose, a ccRCC tissue cohort comprised of patients stratified by response to PD-1/PD-L1-targeted immunotherapy was analyzed by N-glycan and ECM-targeted imaging mass spectrometry (IMS) approaches.

21 ccRCC tissues were categorized by treatment response, where 9 patients responding positively to PD-L1 immunotherapy (>18 months progression-free survival [PFS]) were compared to 12 which had no or very limited response (PFS <6 months). Additional normal kidney tissues were processed as reference samples. Established N-glycan and ECM IMS protocols were used to prepare the formalin-fixed paraffin-embedded (FFPE) tissues, which were then imaged on a timsTOF fleX MALDI-QTOF MS (Bruker). Post-acquisition data was processed in SCiLS Lab software (Bruker) and annotated using existing N-glycan and collagen/ECM peptide databases.

The ccRCC tissues analyzed were obtained from treatment-naïve initial nephrectomy surgery, thus identified N-glycan or ECM biomarkers may predict treatment response. FFPE tissues were initially processed for N-glycan IMS, which detected 120+ N-glycans per tissue. Spectra were collated into a single SCiLS file then co-localized with histopathological annotations. Reference normal kidneys exhibited branched, sialylated N-glycans specific to glomeruli while branched, bisecting, multi-fucosylated species distinguished tubules. In ccRCC tissues, most of the branched, fucosylated and/or sialylated N-glycans detected did not distinguish response classifications. However, putative phosphorylated high mannose N-glycans unique to lysosomal glycoprotein hydrolases were significantly increased in the tumors of non-responders, suggesting a possible metastatic mechanism through secretion and ECM-degradation. Additionally, multiple bisecting N-glycans associated with immune function were statistically associated with the tumors of positive responders, implicating a more active immune response in these tissues. Digestion with collagenase and subsequent ECM IMS revealed 5 ECM peptides significantly correlated with tissues from poor responders. This multiplexed analysis has revealed novel N-glycan and ECM signatures that typify positive and negative responders, with potential to serve as prognosticators of immune-oncological therapeutic response in ccRCC.

(P030) Quantifying Glycan Probability as a Factor of Publication and Reported Glycoprotein Frequency in GlyGen

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Glycans are inherently complex due to diversity in sequence, branching, and linkage arrangements between monosaccharides. The actual diversity is constrained by the rules governing glycan biosynthesis, resulting in features unique to certain glycan types and organisms. This information is potentially useful for interpreting experimental data, but it may not be apparent to non-experts. An approach to lower the barrier to entry for researchers unfamiliar with glycobiology is to encode the expert knowledge of historical structures in forms that are integrable for analysis software. Towards this end, we developed a pipeline to encode glycan probability as a factor of publications and reported glycoprotein frequency, found in the GlyGen database. Using glycan fingerprints, we created a model to generalize probability for any glycan to evaluate agreement with previously reported

structures. Integration of these probabilities into glycan analysis software can help enable automatic interpretation of glycan binding analysis by encoding expert knowledge. Other applications for this system could be to annotate liquid chromatography mass spectrometry datasets and curate submissions to glycan databases.

(P031) Quantifying Glycan Motif Abundance Using Simplified Binding Models Derived from Glycan Array Data

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Glycan binding proteins (GBPs) are broadly used to probe glycan structures in biological samples. The interpretation of GBP binding is generally performed in a manual and qualitative way. The manual approach is challenging and potentially inaccurate due to the inherent complexities in tracking GBP overlapping binding specificities and the changing glycan structures. Software to interpret the binding patterns of GBPs would improve the accuracy of the analysis and would open the door to larger and higher complexity experiments, such as those involving multiple GBPs in parallel or GBP binding after treatment with glycosidases. Our previous approach was limited by oversimplified assumptions of linearity, GBPs having equivalent binding affinity, and insufficient calibration of glycan binding. In this work we introduce a method for solving for glycan motif abundance from the integrated binding patterns of GBPs detected with and without pretreatment with exoglycosidases. We used a simplified competitive binding model for multiple ligands with GBP binding properties derived from glycan array data. We tested the new method on the analysis of 10 glycan binding proteins at 8 enzyme-treatment conditions for the well characterized glycoprotein fetuin. The new method quantified the motif abundance with improved correlation of 0.46 to the motif abundance determined by previous methods. The further validation of the algorithm and incorporation into software that integrates expert knowledge and calibration promises to enable powerful approaches to glycan analysis using GBPs.

(P032) Upregulated expression of the ST6GAL1 sialyltransferase in tumor cells creates sialoglycan ligands for immunosuppressive Siglec receptors

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Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest human cancers. Immune evasion is one of the critical factors for therapeutic failure and poor prognosis. During tumorigenesis, glycans on cancer cells bind to lectin receptors on immune cells, causing immune cell inactivation. In particular, sialic acid–binding immunoglobulin-type lectins (Siglecs) play a crucial role as immune checkpoints in various cancers including PDAC. However, while much research has focused on the effects of Siglecs on immune cell behavior, limited attention has been paid to tumor-associated changes in sialoglycans that serve as ligands for Siglecs. One key glycan change noted in cancer is an increase in the α2-6 sialylation of *N*-glycosylated surface receptors, a modification added by the ST6GAL1 sialyltransferase. ST6GAL1 is upregulated in many cancers including PDAC. Our lab generated a mouse model of PDAC with either pancreas-specific knock-in of oncogenic K-ras (KC mouse) or K-ras in combination with ST6GAL1 knock-in (KSC mouse). Compared to KC mice, KSC mice display accelerated PDAC progression. In the current study, we performed flow cytometry experiments with Siglec-Fc fusion proteins on pancreatic cancer cells with differential ST6GAL1 expression in order to determine whether ST6GAL1 activity creates ligands for Siglecs. We found that the overexpression of ST6GAL1 in 266-6 pancreatic cancer cells induced an increase in sialoglycans recognized by Siglecs 2,3,9 and 10, whereas ST6GAL1 knockdown decreased binding by these same Siglecs. In contrast, ST6GAL1 activity had no effect on the binding of Siglec 1, which is specific for α2-3 sialic acids. Similar results were observed for Suit2 pancreatic cancer cells, and for the HPNE nonmalignant pancreatic acinar cell line. Next, we evaluated Siglec reactivity using a mouse model wherein ST6GAL1 alone was knocked into the pancreas (SC mouse). Analyses of Siglec-Fc binding to acinar cells isolated from WT or SC pancreata corroborated results from established cell lines. Based on these data, we hypothesize that increased α2-6 sialylation on tumor cells promotes dampening of immune response and accelerated PDAC progression via Siglec signaling. Interestingly, with the exception of Siglec 2, Siglecs 3, 9 and 10 are mostly expressed on myeloid cells and macrophages. Moreover, macrophages polarized to an immunosuppressive M2 phenotype are key contributors to PDAC progression. Thus, we conducted immunohistochemistry (IHC) on pancreata from KC and KSC mice, and found an increase in CD163 positive immunosuppressive M2 macrophages in KSC mice. Additionally, IHC using Siglec-Fc proteins revealed that KSC pancreata have higher levels of sialoglycan ligands for Siglec 3, as well as the murine counterparts for Siglecs 9 and 10, Siglecs E and G, respectively. Collectively, these results reveal a potential mechanism for suppressing macrophage cell response in an ST6GAL1-mediated hypersialylated PDAC tumor microenvironment.

(P033) Platelet-Localized ST6Gal1 is not required for murine IgG sialylation *in vivo*

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The antibody subtype IgG forms an important basis of the humoral immune response, and a deeper understanding of this molecule has been recognized as a key to not only protective immunity from pathogens, but also to the reduction of autoimmunity. The regulation of IgG function is driven by IgG subclass (as defined by the heavy chain) as well as the glycan composition at the conserved N297 site of N-glycosylation within the Fc domain. For example, lack of core fucose promotes increased antibody-dependent cellular cytotoxicity, whereas terminal α2,6 sialylation helps to drive immune quiescence. Despite the immunological significance of these compositional changes, little is known about the regulatory aspects underlying IgG glycosylation. Convention states that sialylation of IgG occurs in the *trans*-Golgi network of B cell-derived plasma cells via the sialyltransferase ST6Gal1, but we previously demonstrated that mice with a ST6Gal1-deficient B cell compartment showed unaltered plasma IgG sialylation. This suggests that ST6Gal1 sialylates IgG following its secretion by B cells, leading us to ask where IgG sialylation may be occurring. IgG and ST6Gal1 are both present in platelet granules, which contain proteins from the plasma that can be released during activation, together with a variety of platelet-derived molecules. Having recently ruled out the plasma microenvironment as a predominant site of IgG sialylation, we hypothesized that platelet granules may serve as a reaction vessel for IgG sialylation via either platelet-derived or endocytosed plasma-derived ST6Gal1. Here, we report the derivation of a novel mouse in which platelet factor 4 (PF4)-Cre-mediated ablation of ST6Gal1 alone or in combination with the previously reported hepatocyte-specific knockout of ST6Gal1 was achieved. Analysis of the plasma IgG glycans from these mice revealed a lack of impact upon IgG glycosylation, suggesting that platelet-localized ST6Gal1 is not a major contributor to homeostatic IgG sialylation.

(P034) The impact of α2,6 sialylation upon macrophage polarization and function

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The liver produces many of the glycoproteins found in circulation, and the glycosylation of these proteins plays an important role in modulating their function. As a result of an ongoing effort to determine the site of IgG sialylation in vivo, we previously produced a mouse with a hepatocyte-conditional knockout of ST6Gal1 (HcKO), a sialyltransferase responsible for adding sialic acid residues in an α2,6-linkage to terminal galactose residues of N-glycans on cellular glycoproteins. In previous studies, young HcKO mice exhibited changes in plasma glycoproteins but no overt pathology, although by 40 weeks of age, HcKO mice develop spontaneous fatty liver disease and show increased levels of inflammatory cytokine production. The Cobb laboratory also reported changes in the liver macrophages characteristic of a shift from a normal M2 anti-inflammatory phenotype to a pro-inflammatory M1 phenotype. In the present study, we are working to understand the relationship between sialic acid-containing glycans and the polarization, phenotype, and activity of tissue-resident macrophages, with an emphasis upon how macrophage-expressed Siglecs and their recognition of adjacent tissue cell surface glycans may drive the immunologic effector class.

(P035) Distinct Intracellular Trafficking Restricts IgG Sialylation in Antibody-Secreting Cells

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Immunoglobulin G (IgG) is an antibody subtype associated with anti-inflammatory effects when Fc-domain-localized glycans are α2,6 sialylated. When beta-galactoside α2,6-sialyltransferase 1 (ST6Gal1) was knocked out of B cells in a murine model, the sialylation of surface glycoproteins was found to be decreased, while IgG proteins circulating in the plasma remained sialylated. This suggests that knocking ST6Gal1 out of B cells has different outcomes on glycosylation for different B cell-produced glycoproteins. Here, we found that IgG secreted from murine B cell hybridomas had low sialylation but high α1,6 core fucosylation, mediated by α1,6-fucosyltransferase 8 (FUT8). In contrast, flow cytometry revealed that the surface glycoproteins on the same cells were highly sialylated and poorly core fucosylated. Similar results were found comparing whole cell extracts with isolated IgG by lectin ELISA. Using confocal microscopy, we further discovered that IgG colocalized poorly with intracellular regions high in α2,6 sialylation and ST6Gal1, but colocalized well with regions rich in core fucose and FUT8. This supports a model in which IgG takes a divergent trafficking route intracellularly relative to other glycoproteins to leave the cell without

robust sialylation, thereby providing a possible mechanism for the reason why B cell ST6Gal1 is dispensable for IgG sialylation *in vivo*.

(P036) Use of environmental enzyme screening to identify enzymes that act on post-glycosylation modifications

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Glycans can be modified with diverse chemical groups including sulfate, phosphate, acetyl or phosphocholine residues. Collectively termed 'post-glycosylation modifications' (PGMs), these features increase the structural diversity of glycans and impart a variety of biological functions [1]. For instance, sulfate modification of *N*-glycans is important for the clearance of pituitary hormones and immunoregulation [1, 2]. Yet, the role PGMs play in the cellular glyco-code and how that impacts various biological functions remains under-explored. Characterization of PGMs is in part hampered by a lack of highly defined analytical enzymes that enable their specific detection. Our aim is to identify new PGM enzyme specificities through high-throughput environmental enzyme screening and apply them to glycoanalytical workflows.Here, we report on our established functional metagenomic screening approach to identify enzymes that specifically act upon PGMs. We have initially focused on glycan sulfation, a PGM observed on a variety of glycans including glycosaminoglyans, *N*- and *O*-linked glycans. We first sought enzymes that act upon sulfated *N*-acetylglucosamine (GlcNAc), a common mammalian *N*-glycan sulfation site. We identified a highly specific sulfatase that removes sulfate exclusively from GlcNAc-6-SO_4 when the sugar is in the terminal position on an *N*-glycan, and a sulfate-dependent hexosaminidase that removes intact GlcNAc-6-SO_4 (but not asulfated GlcNAc) from the terminal position of *N*-glycans. Both can be used in structural analyses of *N*-glycans to confirm the presence of terminal GlcNAc-6-SO_4 [3]. In addition, we will present an update on our efforts to screen for enzymes that act upon sulfated galactose (Gal-3-SO_4), another known *N*- and *O*-glycan sulfation site. Finally, we have extended our screening approach to identify enzymes that act upon zwitterionic modifications of glycans (see the abstract by Fossa *et al.*).

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(P037) Natural killer cells treated with kifunensine exhibit increased affinity for IgG and increased antibody-dependent cell-mediated cytotoxicity

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Fc γ receptor IIIa (CD16a) expressed on human natural killer (NK) cells binds IgG to elicit antibody-dependent cell-mediated cytotoxicity (ADCC). In addition to providing a protective role against invading pathogens, FcγR-mediated immunity including ADCC is critical for the treatment efficacy of many therapeutic monoclonal antibodies. Our laboratory previously determined that CD16a with oligomannose *N*-glycans binds IgG1 Fc with a 10-50 fold greater affinity than CD16a with complex-type *N*-glycans. However, it is not known whether increased affinity increases the ADCC response in NK cells. We treated YTS-CD16a cells (an NK cell line) with the mannosidase I inhibitor kifunensine to increase the prevalence of oligomannose-type N-glycans on the NK cell surface. Kifunensine reduced PHA-L staining and increased oligomannose *N*-glycans as determined using MS-based glycomics. Kifunensine-treated YTS-CD16a cells showed comparable CD16a surface expression but a substantial increase in CD16a-mediated antibody-binding affinity at the cell surface. Treatment of YTS-CD16a cells with kifunensine showed a 45% increase in ADCC with using target Raji B cells and the antiCD20 IgG1 rituximab (RTX). We observed a comparable ADCC increase by treating primary human NK cells with kifunensine. A complete blockage of ADCC occurred by blocking either treated or untreated cells with an anti-CD16a Fab (3G8), indicating that CD16a is indispensable for the increased ADCC activity. Lastly, we observed an additional 25% higher ADCC for the kifunensine treated cells stimulated by afucosylated RTX when compared to the untreated control. These data demonstrate that treating primary and cultured NK cells with kifunensine increased both antibody-binding affinity and the efficacy of an important immune response.

(P038) SARS-COV-2 RETAINS GLYCOSYLATION SITES OF THE SPIKE PROTEIN ACROSS THE VARIANTS OF CONCERN, BUT GLYCOSYLATION PROFILES AT DIFFERENT SITES DISPLAY CHANGES.

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The glycosylation of the spike (S) protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19, modulates the viral infection by altering conformational dynamics, receptor interaction and host immune responses. Several variants of concern (VOCs) of SARS-CoV-2 have evolved during the pandemic and crucial mutations on the S protein of the virus leading to increased transmissibility and immune escape were reported. In the present study we aim to evaluate the significance of glycosylation across the variants of SARS-CoV-2 by characterizing the site-specific glycosylation changes on the S proteins on VOCs. In this study, we applied state-of-the-art glycoproteomics and glycomics techniques to evaluate the N-and O-glycosylation of the S proteins of wild type and five VOCs of SARS-CoV-2 such as B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), B.1.1.529 (Omicron), produced under the same conditions. Interestingly, the N-glycosylation sites on the S protein is highly conserved among the reported spike mutant variants, particularly at the sites on the receptor binding domain (RBD), including omicron. Our detailed profiling of the glycosylation at each of the individual sites of the S protein across the variants revealed intriguing association of glycosylation pattern on the variants. We observed that about half of the N-glycosylation sites of S protein displayed altered distribution of glycans across the variants. The variants which showed higher resistance to neutralizing antibodies displayed a decrease in the relative abundance of complex-type glycans and an increase in oligomannose glycans. The glycosylation sites with significant changes were observed at both the N-terminal domain (NTD) and RBD domain of the S protein of VOCs. O-glycosylation at the RBD domain showed lower occupancy in the variants in comparison to the wild type Wuhan strain, nevertheless all variants retained the O-glycosylation in site T323. Our study on the comparison of glycosylation pattern of the SARS-CoV-2 variants would shed light to understand how the virus evolves itself to trick the host immune system and alter its infectivity. Our study also paves way for future research to understand the key roles played by both N- and O- glycosylation in SARS-CoV-2 infection, as all glycosylation sites are highly conserved by the virus across all variants, including the highly mutated Omicron variant.

(P039) Comparative Glycomics and Glycoproteomics of SARS-CoV-2 variants B.1.1.529 and BA.2 Spike Proteins

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Since late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been an ongoing worldwide pandemic. Since the original wild type Wuhan-Hu-1 (WT) strain was identified, many different variants have emerged. Of these, variants with an increase in transmissibility, infectivity and/or mortality are deemed as variants of concern (VOC). The Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1) and Delta (B.1.617.2) variants were all deemed VOCs and at one point were the dominant SARS-CoV-2 variant. However recently, the Omicron (B.1.1.529) variant and its descendent lineages (BA.1, BA.1.1, BA.2, BA.3, BA.4, BA.5) have become the dominant strains in recent months. To determine glycosylation changes of B.1.1.529 and the BA variants, we have performed comprehensive glycomic and glycoproteomic analysis of the B.1.529 and BA.2 variants spike (S) proteins. Across both variants, all 22 N-glycosylation sites identified in the WT are conserved. This is note-worthy as there are over 30 amino acid mutations in the Omicron variant, none of which effect the N-X-S/T consensus sequence. This is also noted in the previous VOCs, where all N-glycosylation sites were conserved, excluding one site in B.1.617.2. The B.1.1.529 S protein analyzed contains 30 amino acid mutations, 3 amino acid insertions and 6 deletions. The BA.2 S protein analyzed contains 28 amino acid mutations and 3 deletions. Interestingly, though the sites of glycosylation are conserved, the glycosylation profile of these sites has changed.

Our preliminary glycomics data shows that the B.1.1.529 S protein and the BA.2 S protein have different N-glycosylation patterns. Based on glycomics analysis, B.1.1.529 has a higher abundance of Oligomannose-type glycans compared to BA.2. The BA.2 spike protein in contrast has a higher abundance of complex/hybrid type glycans, specifically more complex glycans containing both fucose and sialic acid. O-glycosylation of the SARS-CoV-2 S proteins is low, however like N-glycosylation sites, O-glycosylation sites are conserved between the B.1.1.529 and BA.2 S proteins. O-glycosylation at T323 has been reported across many S protein analyses by different research groups, both in the WT strain and the subsequent variants. This could indicate that O-glycosylation at this site may be imperative for host cell infection. Preliminary glycoproteomics results indicates the B.1.1.529 S protein has lower N-glycan occupancy than the WT S protein. We hypothesize that the increase in unoccupied

N-glycan sites may correspond to the increased transmissibility and infectivity of the Omicron variants. Understanding the variation in S protein glycosylation in the SARS-CoV-2 variants could help shed light on the changes in infectivity, and could play an important role in developing vaccines and therapeutics aimed at targeting these mutated S proteins.

(P040) Glyfinder and Glycoprotein Builder: Online Tools for Finding and Modeling Glycoproteins in the PDB

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Tens of thousands of 3D structures of oligosaccharides have been deposited into the Protein Databank (PDB), representing hundreds of thousands of hours of effort by crystallographers. Yet, despite the critical importance of these structures in furthering the development of glycomimetic drugs, in explaining the activity of glycan-processing enzymes, and in providing a deeper understanding of the properties of glycoproteins and vaccines, they remain unnecessarily difficult to locate within the PDB. Part of this is due to limitations in searching for oligosaccharides on the PDB website, even after a recent carbohydrate remediation project completed by the PDB. While several databases have been reported that contain carbohydrate structural information extracted from the PDB, few offer flexible search capabilities and even fewer provide independent assessment of data quality. Here we present the GlyFinder and GlyProbity webtools (glycam.org/gf) and illustrate their application to locating oligosaccharides, carbohydrate derivatives, and glycoproteins stored in the PDB. We highlight the utility of curating the data on the basis of the theoretical conformational (CHI) energies [1] of the glycosidic linkages and illustrate how the deposited data can be employed to generate 3D models of glycoproteins, including the SARS-CoV-2 Spike protein [2].

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(P041) Glycosaminoglycans direct extracellular ST6GAL1 bioactivity in human monocytic cells

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The sialyltransferase ST6GAL1 mediates the transfer α2,6-linked sialic acid to Gal(β1,4)GlcNAc termini of glycans. In addition to acting within the Golgi, a biologically impactful exogenous circulatory pool of ST6GAL1 derives from sources including liver, cancer exosomes and cancer nanoparticle exomeres. Exogenous ST6GAL1 modulates monocyte-macrophage development, B cell development, IgG and granulocyte production, and enhances tumor cell proliferation and invasiveness. Considering the well-established role of glycosaminoglycans (GAGs) and proteoglycans (PGs) in growth, proliferation and invasion of cancer cells, we reasoned that these biopolymers may modulate the function of ST6GAL1. Computational predictions using combinatorial virtual library screening algorithm indicated that ST6GAL1 preferentially recognizes heparan sulfate sequences among other GAGs including dermatan sulfate (DS) and chondroitin sulfate (CS). Fluorescence-based affinity studies on ST6GAL1 binding to HS, DS, CS and their oligosaccharides confirmed these predictions. The ability of GAGs to modify ST6GAL1 enzymatic activity directed at asialofetuin was examined. "Long-chain" heparin (ave MW 18KDa) was the most effective in attenuating ST6GAL1 enzymatic activity against asialofetuin, with EC_{50} of ~10nM. Interestingly, "short-chain" heparin oligosaccharide (ave MW 1,800 Da) was significantly less effective (EC_{50} ~ 6 mM) as was heparan sulfate (MW ~17kDa-68kDa) with an EC_{50} ~ 2.4mM (based on ave MW). In contrast, dermatan sulfate (MW 1488) and chondroitin sulfate (MW 1488) were essentially ineffective in suppressing ST6GAL1 enzymatic activity; partial inhibition was observed only at the highest concentration used (~700 mM). We previously reported the ability of exogenous ST6GAL1 to interact with human THP-1 monocytes to elicit wholesale changes in gene transcription profiles. Here, using formalin-fixed THP-1 cells, we show that long-chain heparin, at 28 mM concentration, affected a 50% inhibition of sialylation by exogenous ST6GAL on the THP-1 cell surface. In total, our results implicate a role for GAGs as co-factors in directing exogenous ST6GAL1 cellular interactions.

(P042) Engineering Homogenous Glycoforms of Differentially Sialylated Blood Glycoproteins Bearing Discrete Bi-Antennary or Multi-Antennary N-Glycans for Discovering the Molecular Nature of Physiological Ligands Regulated by the Ashwell-Morell Receptor

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Mechanisms determining the half-lives of blood proteins have been difficult to perceive. In the simplest case, the rate of elimination from the bloodstream is determined by proteinaceous receptor-ligand binding and endocytic uptake. However, most proteins present in the blood do not have corresponding endocytic receptors that recognize their protein sequences. That would require many hundreds if not thousands of dedicated clearance receptors, and for the vast majority of circulating blood proteins, that does not appear to be the case. Instead, most secreted proteins are post-translationally modified with N-glycans, which when remodeled by glycosidases at different rates in circulation can then be recognized by endocytic lectin receptors expressed among the vasculature and parenchyma of multiple organs. The two most abundant N-glycan termini of blood glycoproteins are sialic acid linked to galactose in α2-3 or α2-6 linkage. Endogenous neuraminidases present in blood can remove one or both types of sialic acid linkages to expose the galactose thereby rendering a preferred ligand of the prototypical endocytic lectin the Ashwell-Morell receptor (AMR) of the liver, that clears them from circulation. Whether Siaα2-3Gal and Siaα2-6Gal forms of specific N-glycoproteins exhibit different rates of desialylation and engagement with the AMR is not well understood and have primarily been studied using synthetic neoglyconjugates. Here we use glycoengineered CHO cells to produce a set of human alkaline phosphatases (IAP and TNAP) carrying multi- or bi-antennary N-glycans capped exclusively with α2-3 or α2-6 linkages of sialic acid (Neu5Ac). We are now comparing the circulatory half-lives of these glycoproteins *in vivo* among wild-type mice as well as littermates lacking the Asgr1 or Asgr2 protein subunit of the AMR. Data collected thus far indicates that neither Siaα2-3Gal nor Siaα2-6Gal are ligands of the AMR unlike their desialylated preparations. The impact of these sialic acid linkages and their rates of hydrolysis in AMR-dependent clearance, as well as the impact of N-glycan branching, are being investigated. Our findings will reveal the structural basis of physiological AMR ligands *in vivo* among non-denatured blood glycoproteins.

(P043) Engineering of Lectenz®, a novel class of high-affinity reagents for the detection and enrichment of specific carbohydrate structures

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Introduction: Glycans containing sialic acids have long been implicated as biomarkers for a variety of diseases, including endocrinal, oral, and colon cancers. As the investigation of the dynamic changes in the levels of glycoforms becomes more commonplace, specific glycan binding proteins are essential to overcome current limitations in the discovery and exploitation of disease-related glycans.

Methods: Using computationally-guided design and directed evolution, we have been converting carbohydrate-processing enzymes into catalytically inactive affinity reagents with tunable specificities. These novel LECTin-like, ENZyme-derived reagents have been shown to offer numerous advantages over other carbohydrate-binding reagents: they are high affinity, yet retain the exquisite substrate specificity of the parent enzyme, they can be cost-effectively produced, and they can be used as capture reagents for enrichment applications.

Results: Currently, we have developed and commercialized two sialic acid-binding Lectenz® engineered from a neuraminidase, pan-specific and α2,3-specific, as well as an α2,6-specific SiaFind™ reagent engineered from a *Polyporus squamosus* lectin. They have demonstrated in a number of applications such as Western blotting, flow cytometry, immunohistochemistry, and affinity chromatography. These applications validate the simplicity, robustness, and accuracy of SiaFind™ reagents in glycoscience research. Supported by NIH grants R44OD024964, R43GM135984, and R43GM136013.

(P044) Exploring the effects of tissue structural cell and tumor cell heparan sulfate on immune system trafficking, activation, and intercommunication via amphiregulin

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Heparan sulfate (HS) is ubiquitously present on the surface of structural tissue cells and tumor cells during homeostasis, disease, and cancer, but how it affects immune cell trafficking, activation, and signal transmission to tissue and tumor cells in these

contexts is poorly understood. Furthermore, amphiregulin (Areg), a growth factor used by immune cells for intercommunication with structural tissue and tumor cells, contains a HS binding domain. We investigated the downstream signaling modalities of Areg-mediated cell activation in the context of HS suppression. Areg signaling is qualitatively altered, but still present, upon exogenous inhibition or genetic ablation of HS, changing from high levels of Akt and Erk phosphorylation to lower but maintained Akt phosphorylation and similar levels of Erk phosphorylation. We further probed the consequences of HS-dependent vs. -independent Areg signaling at the transcriptional level. Next, we used a panel of CRISPR-Cas9-mediated knockout Lewis Lung Carcinoma (LLC) cell lines to interrogate how various aspects of HS (core proteoglycan, HS chain extension, sulfation status) contribute to Areg signaling. We found that the presence of the glypican family of HS-harboring proteoglycans on the surface of cells is critical for Areg signaling. Lastly, we explored the effects of cancer cell HS on immune system trafficking to and activation within tumors. HS-deficient tumors experience similar levels of lymphoid cell trafficking and activation, but show a significant increase in the presence of certain myeloid cell type (neutrophils, monocytes, and macrophages). Additionally, tumor-associated macrophages within HS-deficient tumors show an altered phenotype, with increased maturation markers. Overall, our work has illuminated previously understudied aspects of the relationships between HS, Areg, and the immune system.

(P045) Impact of mitochondrial glucose and glutamine energy metabolism on hyaluronan in asthmatic human airway smooth muscle cells

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Alteration of cellular energy metabolism impacts metabolic processes in diseases, including asthma. We showed that asthmatics had ~100 kcal/day higher resting energy expenditure than control subjects, and platelets isolated from asthmatics depend more on mitochondrial respiration and less on glycolysis. These data suggest that cellular energy metabolism is altered in asthma, which may link to asthma pathophysiology. Airways in asthma undergo structural remodeling due to hyperproliferation of airway smooth muscle cells and excessive deposition of hyaluronan (HA). Glucose and glutamine are both essential nutrients for energy production and biomolecule synthesis in proliferating cells. Glucose entering glycolysis is converted into acetyl CoA, while glutamine is converted into glutamate by glutaminase (GLS), which then enters mitochondria for energy production. Glucose and glutamine are the building blocks for HA synthesis. Despite the importance of glucose and glutamine on energy metabolism and HA biosynthesis, our knowledge on how bioenergetic metabolism of glucose and glutamine influences HA synthesis in asthma is unclear. We hypothesize that this bioenergetic metabolism regulates HA synthesis in asthma. To test this, we evaluated bioenergetics and HA contents of human airway smooth muscle cells (HASMCs) isolated from lungs of asthmatic (n=5) and non-asthmatic, control (n=5) subjects. Bioenergetic glucose and glutamine metabolism were inhibited by UK5099, the mitochondrial pyruvate carrier inhibitor, and by BPTES, the glutaminase inhibitor, respectively. Asthmatic HASMCs have higher hyaluronan synthase 3 ($p<0.01$) and glutaminase ($p<0.04$) proteins than controls. We did not find differences in glucose and glutamine dependency for mitochondrial respiration between control and asthmatic HASMCs. However, both control and asthmatic HASMCs have higher dependency on glutamine (64%) than on glucose (21%) for mitochondrial respiration, suggesting that glutamine is an essential energy source. Inhibition of mitochondrial glucose bioenergetics decreased spare respiratory capacity (SRP) in asthmatic ($p=0.006$), but not in control HASMCs, while glutamine bioenergetics inhibition decreased SRP in both control ($p=0.06$) and asthmatic ($p=0.005$) HASMCs. Inhibition of the mitochondrial glucose and glutamine oxidative pathway decreased HA in both control (glucose $p=0.03$; glutamine $p=0.08$) and asthmatic (glucose $p=0.08$; glutamine $p=0.02$) HASMCs. Decreases in HA were accompanied by changes in proteins involved for energy metabolism and in the HA pathway. We conclude that bioenergetic metabolism of glucose and glutamine is essential for HA synthesis in both control and asthmatic HASMCs.

(P046) Comprehensive Glycomic Characterization of Ewing's Sarcoma to Identify EWS Tumor Specific Glycan Epitopes

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Glycosylation is a common modification for proteins and lipids that can impact their structure and function. In human cells, the two major forms of protein glycosylation that occur on most transmembrane and secreted proteins are N-linked glycosylation (N-glycans) and O-linked glycosylation (O-glycans). Glycosylation is often qualitatively and quantitatively altered

by disease states. In tumor cells, the altered glycans are termed tumor-associated carbohydrate antigens (TACAs), with common examples including Tn antigens on O-glycoproteins and the glycolipid GD_2. Importantly, many biomarkers for tumor screening or diagnosis are either altered glycoproteins or TACAs. TACAs can also be therapeutic targets. Although GD_2 was identified as a biomarker for immunotherapy for neuroblastoma in children, few biomarkers have been identified for other pediatric tumors. Ewing sarcoma (EWS) is a rare cancer of bone or soft tissue typically occurring in teenagers and young adults. EWS has a characteristic genetic alteration: rearrangement of the EWS gene on chromosome 22q12 with an ETS transcription factor gene to form the chimeric *EWS-FLI* gene in 85-90% of cases. However, little is known about the glycomics of EWS cells. We performed a comprehensive investigation of EWS tumor-specific glycan epitopes by analyzing human-derived EWS cell lines and EWS human tissue samples with multiple techniques including molecular biology, biochemistry, glycobiology, mass spectrometry (MS), ultra performance liquid chromatography, and next-generation sequencing. MS analysis of N-glycans revealed that 12 of 17 EWS cell lines contained unique core fucosylated paucimannose (Man3F) N-glycans with >5% relative abundance. O-glycans in one EWS cell line had novel oligo-sialic acid structures, which were absent from corresponding pre-treatment cells. Three of six EWS cell lines analyzed expressed Tn antigens on their surface. The preliminary glycolipid characterization with high-performance thin-layer chromatography (HPTLC) and MS analyses showed EWS cell lines had variable glycolipid profiles. TLC results for most of the cell lines tested indicated the presence of cerebrosides, ceramide trihexoside, GM_2, and GM_3. EGCase I reactions were analyzed by MS, and showed features consistent with glycans released from GM_2, GM_3, $GD_{1a/b}$, and other monosialylated and neutral glycan species. Additionally, four of eight cell lines analyzed expressed GD_2, as detected by flow cytometry with the anti-GD2 antibody, dinutuximab. In-depth glycomic characterization and other molecular analyses of EWS lines and mesenchymal control cells are ongoing. The altered glycans identified in EWS cell lines, including Tn antigens and GD_2, will also be validated and confirmed in the EWS tissue specimen with the goal to identify unique glycan structures or TACAs as biomarkers and potential targets for developing EWS immunotherapies.

(P047) Collaboration, Service and Trainings at the Complex Carbohydrate Research Center

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For more than 30 years, the Complex Carbohydrate Research Center (CCRC) has collaborated with universities, federal agencies, and industry from the US and other countries on detailed structural characterization of glycoconjugates. We have expertise in a full range of analytical methodologies needed to conduct comprehensive research in glycobiology. We are equipped with the state-of-the-art instrumentation including a Thermo Orbitrap-Eclipse MS, Thermo Orbitrap-Fusion MS, Waters Synapt XS MS, Bruker RapiFlex MALDI-MS, AB SCIEX TOF/TOF 5800 MALDI-MS, SPR, multiple analytical and preparative separations options, and GC-MS. The CCRC's NMR facility offers outstanding resources for high-field NMR spectroscopy including 900 MHz, 800 MHz, three 600 MHz and a 500 MHz spectrometer. These spectrometers are equipped with variety of probes including a HRMAS probe for solid state analysis and several H-C/H-N cryoprobes and HCN cryoprobes dedicated to biomolecular NMR applications.

Collaborative projects with the CCRC can be very diverse and can include: Glycomics and glycoproteomics, isolation and analysis of Glycosaminoglycans (GAGs) and GAG-derived products, polysaccharides and Lipopolysaccharides (LPS), peptidoglycans, and glycolipids. Staff at the CCRC-AST also excel in the development of novel characterization and isolation methods, depending on the individual client's needs.

The CCRC offers yearly training courses on **techniques for structural characterization glycans of glycoproteins, glycolipids, polysaccharides, and GAGs, as well as courses on mass spectrometry techniques and software analysis.** Training participants will perform hands-on experiments and analysis by mass spectrometry, GC/MS, HPLC-UV and fluorescent detection, HPAEC-PAD, lectin blotting, and thin layer chromatography. The hands-on experience is further enhanced by lectures from faculty on various fields of glycobiology.

(P048) Spatially resolved isotopic labeling to study glycogen and N-glycan flux in situ by MALDI mass spectrometry imaging

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Glucose hypometabolism is considered a critical and actionable clinical hallmark of Alzheimer's Disease (AD). Glucose participates in the anabolic processes of glycogen and N-glycan metabolism, which are found in virtually all organs. Our lab

previously discovered increased glycogen and N-glycans in unique regions of both mouse and human AD brains. Mechanisms behind such striking phenotypes remains undefined. Herein, we report the ability to image isotopically labeled glycogen and N-glycans to study their flux in a spatial context. Mice were provided a special diet that contained $^{13}C_6$-glucose as the primary carbohydrate source administered ad libitum for up to 3 days. At the experimental endpoints, mouse brains were formalin-fixed followed by multiplexed glycogen and N-glycan analyses using traveling wave ion mobility mass spectrometry imaging analyses. We discovered robust, regional-specific labeling patterns of glycogen and N-glycans in the central nervous system of AD and wild-type (WT) mouse models. Labeling patterns for glycans and glycogen were heterogenous and localized to regions our lab has previously discovered as accumulation points for complex carbohydrates in AD. Interestingly, we found similar incorporation of labeled glucose into glycogen in the cortex of AD and WT animals. Conversely, isotopic labeling of N-glycans in 5xFAD mice was significantly increased in the first 24 hours compared to WT mice. These studies suggest a dysregulation in N-glycan biosynthesis in a mouse model of beta-amyloid accumulation and dementia; however, the pathophysiological glycogen accumulations seen in AD patients could be due to other storage defects which warrant further investigations.

(P049) Carbohydrate Antigens Induce Immune Evasion Mechanisms in a Murine Breast Cancer Model

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Treatments for advanced stage breast cancer remain largely ineffective due to the ability of the tumor to grow unimpeded through immune suppression and evasion mechanisms. Many tumors, including breast tumors, are known to express aberrantly glycosolylated antigens on cell surface proteins. Tn is a tumor-associated carbohydrate antigen (TACA) previously known to be associated with immune suppression in helminth infections. One ligand for Tn is CD301 (also known as MGL), which is expressed on certain dendritic cell and macrophage populations of immune cells in humans (CD301b/MGL2 in mice). Both Tn and its ligand CD301 have been linked with immune suppression; however, their roles in breast cancer has not been well described. We hypothesized that breast cancer cells use the Tn-MGL signaling pathway as a mechanism to induce immune suppression and avoid immune clearance. We have investigated the association of Tn and CD301b immune cells with immune suppression and concomitant breast tumor growth in a mouse model. We have identified a previously uncharacterized $B220^+CD19^+CD301b^+$ immune cells which accumulate within the breast tumor in mouse models. These cells express extracellular markers associated with regulatory B cells, and express high levels of IL-10, indicating a suppressive immune cell population. We observe these cells specifically within the breast tumor microenvironment and show they are enriched in both cell-line derived and spontaneous tumors expressing higher levels of Tn antigen through aberrant glycosylation pathways. Ablation of these $CD301b^+$ cells in a DTR mouse model significantly reduces Tn-high tumor growth and reduces accumulation of $CD11b^+Gr^+$ cells, commonly considered myeloid derived suppressor cells. Inhibition of this Tn-CD301b interaction is a promising target for overcoming immune suppression and stimulating the immune response against breast cancer cells.

(P050) Novel role for the ST6Gal1 sialyltransferase in pancreatitis

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Pancreatitis is a life-threatening disorder characterized by pancreatic acinar cell death, inflammation and fibrosis. In an attempt to heal the damage, a subset of acinar cells de-differentiates toward a ductal, progenitor-like phenotype in a process known as acinar to ductal metaplasia (ADM). Cells undergoing ADM are able to re-enter the cell cycle, facilitating tissue regeneration. Studies by our group suggest that the sialyltransferase ST6Gal1 is involved in promoting ADM. ST6Gal1 adds an α2,6-linked sialic acid to N-glycosylated receptors, thereby regulating cell signaling and phenotype. In the current study, we report that ST6Gal1 is robustly expressed in the tissues of patients with chronic pancreatitis and mice with experimental pancreatitis, whereas ST6Gal1 is undetectable in normal acinar cells. Moreover, ST6Gal1 is selectively expressed in the ADM-like cells, as identified morphologically and by staining for known ADM markers. Immunofluorescent staining of tissues revealed that most of the ST6Gal1-positive cells co-expressed Ki67, confirming a proliferative state. To investigate a functional role for ST6Gal1 in ADM, we developed transgenic mice with ST6Gal1 expression in the pancreas ("SC" mice). Significantly, acinar cells from SC mice exhibited progenitor-like features, including enriched expression of stem and ductal genes, even in the absence of pancreatitis. Upon placement of acinar cells into 3D culture, SC cells formed more ADM-like morphological structures than WT cells. We also examined ADM using organoid models, which revealed that SC-derived organoids had increased expression of stem and ductal genes relative to WT organoids, as well as greater organoid-forming potential and growth. To evaluate inflammation-induced ADM, SC and WT mice were injected with cerulein to stimulate pancreatitis, and the ADM-like cells were quantified

by flow cytometric analyses of ADM surface markers. Following induction of pancreatitis, more ADM-like cells were found in SC vs. WT pancreata. In other studies, we interrogated the mechanism by which pancreatitis induces ST6Gal1 expression. We treated pancreatic cell lines with a range of pro-inflammatory cytokines secreted by immune cells during pancreatitis, and determined that IL-6 and IL-1β stimulated an upregulation in the "YZ" isoform of ST6Gal1, which is driven by the P3 promoter. We also verified high levels of IL-6 and IL-1β in pancreatitis tissues. The ST6Gal1 P3 promoter has multiple binding sites for STAT3 and NFκB, which are transcription factors activated by IL-6 and IL-1β, respectively. Importantly, inhibitors of STAT3 and NFκB blocked cytokine-induced ST6Gal1 expression, implicating the IL-6/STAT3 and IL-1β/NFκB cascades in ST6Gal1 upregulation. Taken together, these results highlight a novel molecular pathway by which the cytokine-mediated upregulation of ST6Gal1 in acinar cells reprograms the cells to adopt progenitor-like characteristics necessary for pancreatic regeneration.

(P051) Platelet and Myeloid Cell Phenotypes in a Rat Model of Fabry Disease and the Role of Glycosphingolipids in Sensitizing Platelets to Agonist-induced Activation

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Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal enzyme α-Galactosidase-A (α-GalA). Fabry disease results from the widespread accumulation of the glycosphingolipids (GSLs) globotriaosylceramide (Gb3), and globotriaosylsphingosine (lyso-Gb3), impacts multiple organ systems and leads to impaired quality of life. Thrombotic events are common, with strokes and heart attacks contributing to a shortened lifespan for male and female Fabry patients. Previously we showed GSL accumulations in the bone marrow, circulation, and platelets from male α-Gal A-deficient rats contribute to increased platelet activation in response to agonists. However, the extent that these GSLs accumulate in the female Fabry population and the mechanisms by which Gb3 and lyso-Gb3 increase thrombotic risk are incompletely defined. Using a rat model of Fabry disease, we aim to improve our understanding of GSL accumulation among female animals and define the mechanisms linking GSL accumulation to thrombotic disease. Using a flow cytometric assay quantifying fibrinogen binding to the integrin GPIIb/IIIa, we assessed the activatability of platelets after treatment with the platelet agonist ADP. In contrast to Fabry male rats, we found that α-GalA-deficient female rats do not present with an increased platelet activation response to ADP. Notably, in the bone marrow of 52-week-old female Fabry rats, histological staining of femur sections with *Griffonia simplicifolia isolectin* B4 (IB4), a lectin with high affinity for terminal α-galactose, revealed striking accumulations of GSLs. Further staining with CD68 and CD3 showed elevated levels of monocytes and lymphocytes, consistent with what is seen in the circulation. Additionally, complete blood counts (CBCs) revealed that at 15 weeks, homozygous Fabry female rats had significant increases in lymphocyte and monocyte counts compared to wild-type (WT) females. This trend is seen from 15-75 weeks of age and indicates that homozygous Fabry females have chronically high levels of white blood cells in their circulation. Additional CBC data showed a significant difference in the mean platelet count of WT and homozygous Fabry females at 50-75 weeks. Lastly, we assessed the ability of α-GalA substrates to sensitize platelets to activation using WT male and female platelets from 50–75-week animals by incubating WT platelets with various GSLs and concentration ranges. WT platelets incubated with 10 μM Gb3 and 2 μM lyso-Gb3, GSLs that accumulate in Fabry patients, as opposed to 10 μM glucocerebroside and 2 μM lyso-glucocerebroside, significantly increased platelet activation and binding to fibrinogen, even without ADP. Together, these data suggest that chronically high concentrations of the Fabry-associated GSL, Gb3 directly contribute to an increased level of baseline platelet activation even in the absence of exogenous platelet agonists. (NIH K12HL141954 to AK and NMD, R01DK042667 to NMD)

(P052) ST6GAL1 insufficiency suppresses hematopoietic recovery from acute bone marrow radiation syndrome

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Acute radiation syndrome (ARS) originates via exposure to harmful level of radioactive stimuli driving organ tissue failure and eventual mortality. Organ systems most sensitive to irreversible radiation injury include: (1) the gastrointestinal system, where mortality ensues days after lethal dose irradiation and (2) the hematopoietic system, whereby mortality eventually develops from the inability to replenish multilineage blood cells populations. Previously we have showed that the inability to express functional ST6GAL1, which mediates α2,6-sialylation of Gal (β1,4) GlcNAc glycan termini, renders increased sensitivity to ionizing radiation in mouse models. Mortality of *St6gal1*-null animals, by radiation resulted from irreparable damage to the gastrointestinal tract, putatively to the intestinal stem cells necessary for the continuous regeneration of the gut epithelium.

While clear that hematopoiesis is also damaged by radiation, it is not known whether functional ST6GAL1 protects the marrow from radiation injury. Here, we evaluate how ST6GAL1 contributes to the earliest events in hematopoiesis following irradiation *in vivo*. Mice compared between age/sex matched controls, were exposed to a reduced radiation regiment (3 Gy) and observed for 5 days post irradiation. At baseline, similar cell numbers of hematopoietic progenitors defined as lineage^{neg} ; c-KIT^{pos} ; Sca-1^{pos} (LSK) were present in *St6gal1*-null and control *wild-type* C57BL/6 marrow. However, 5 days after exposure to 3Gy irradiation, we observed a 2-fold reduction in LSK numbers in the *St6gal1*-null compared to the wild-type marrow. LSK cells from wild-type marrow had strikingly increased proportion of SNA^{pos} cells, strongly suggesting involvement of ST6GAL1 during re-establishment of hematopoietic homeostasis. The LSK pool was further dissected to examine the contributing subpopulations including long-term and short-term hematopoietic stem cells (LT-HSC, ST-HSC), and multipotent progenitors (MPP). We observed significant decreases in cell numbers of MPP and ST-HSCs of the *St6gal1*-null mouse. Interestingly, LT-HSCs did not demonstrate significant changes in cell count between wild-type and the *St6gal1*-null mouse suggesting non-reliance on native ST6GAL1 expression for that specific marrow compartment. In addition, 40 days after exposure to 3Gy radiation, *St6gal1*-null mice had a trending reduction in circulating white blood cells (lymphocytes, monocytes, and granulocytes), not observed in wild-type. Marrows were flushed from *St6gal1*-null and wild-type mice at baseline and cultured *in vitro*. The cultures were then irradiated with 3 Gy dosage. Cell proliferation 5 days after irradiation *ex vivo* was monitored, and there was significantly reduced proliferation in *St6gal1*-null cultures compared to wild-type. Together, our data point to a role for functional *St6gal1* in damage restoration of not only the gastrointestinal system, but the hematopoiesis system, after radiation injury.

(P053) Immune complexes containing aberrantly O-glycosylated IgA1 isolated from sera of patients with IgA nephropathy activate multiple protein-tyrosine kinases in cultured mesangial cells through receptor transactivation

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IgA nephropathy (IgAN) is an autoimmune disease driven by pathogenic immune complexes formed in the circulation and depositing in the kidneys. Circulating immune complexes (CIC) in patients with IgAN consist of galactose-deficient IgA1 (Gd-IgA1) bound by Gd-IgA1-specific IgG autoantibodies. These CIC induce cellular proliferation of cultured primary human mesangial cells (MC). This process involves activation of multiple protein-tyrosine kinases. We previously found that Gd-IgA1-IgG-containing CIC added to MC in culture associated with integrin $\alpha1\beta1$ and $\alpha5\beta1$. Here, we assess the relationship between integrins and activated protein-tyrosine kinases.

Gd-IgA1-containing CIC were isolated by size-exclusion chromatography from sera of patients with IgAN. Quiescent cultured MC were incubated with CIC for 15 min at 37°C, either without or with various individual inhibitors, including obtustatin (integrin $\alpha1\beta1$ inhibitor), RGD (integrin $\alpha5\beta1$ inhibitor), and dasatinib (broad-spectrum protein-tyrosine kinase inhibitor). Cells were then washed and lysed. The lysates were analyzed directly or used for immunoprecipitation (IP). Cell lysates were analyzed by SDS-PAGE-immunoblotting to detect phosphorylated and total ERK1/2, AKT, Axl, and PDGF receptor β (PDGFR-β). IP of integrin $\alpha1\beta1$, integrin $\alpha5\beta1$, or PDGFR-β was performed using monoclonal antibodies specific for integrin $\beta1$ (HUTS21, P5D2) or an antibody specific for PDGFR-β. The IP products were then analyzed using SDS-PAGE-immunoblotting with antibodies against phosphorylated and total integrin $\beta1$ and PDGFR-β.

Gd-IgA1-containing CIC added to MC induced phosphorylation of cellular ERK1/2, AKT, Axl, PDGFR-β, and integrin $\beta1$. Phosphorylation of these proteins was inhibited by integrin inhibitors (obtustatin, RGD) and dasatinib. As PDGFR-β and integrin $\beta1$ are membrane receptors, we tested whether they may be physically associated, either constitutively or upon CIC activation of MC. In MC incubated with CIC but not in control MC without CIC, phosphorylated integrin $\beta1$ was pulled down with antibodies against PDGFR-β. Conversely, PDGFR-β was pulled down with antibodies specific for integrin $\beta1$, irrespective of whether CIC or inhibitors were used. These results revealed physical association of these integrins with PDGFR-β and CIC-induced crosstalk of activated integrin $\beta1$ with PDGFR-β.

Therefore, CIC activation of integrins $\alpha1\beta1$ and $\alpha5\beta1$ results in transactivation of PDGFR-β and further downstream signaling. This process may resemble the transactivation of Axl by PDGFR-β we described previously for PDGF. Better understanding of processes induced in MC by CIC will inform development of future disease-specific therapeutics for IgAN.

(P054) Galectin-3 disrupts the airway epithelial integrity during influenza A virus infection

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Influenza A virus (IAV) infects the airway and alveolar epithelia in humans, and in severe cases causes acute respiratory distress syndrome (ARDS) resulting from increased alveolar permeability due to the disruption of cell-cell tight junctions. The detailed

mechanisms involved, however, remain to be fully elucidated. Galectins are β-galactose-binding lectins implicated in diverse cellular functions. In previous studies on a mouse model, we showed that influenza infection enhances galectin-3 secretion in the bronchoalveolar fluid. In the present study, we investigated *in vitro* the potential role of this secreted galectin-3 in airway epithelial integrity during IAV infection. Our results indicate that 8 hours post infection the surface of model airway epithelial cells (A549) is significantly desialylated, exposing the sub-terminal β-galactose ligands, with a concomitant increase in the binding of recombinant galectin-3 (rGal3). We detected potential galectin-3 receptors β1-integrin, CD147 and MUC1 on the surface of A549 cells, and observed an increase in the secretion of matrix metalloproteinases MMP2 and MMP9 after exposure of these cells to rGal3. Lastly, exposure of A549 cell monolayer to rGal3 disrupted the surface distribution of tight junction proteins occludin and ZO-1, while also increasing the monolayer permeability. Therefore, we propose that during IAV infection, galectin-3 secreted into the airway contributes to the disruption of cell-cell tight junctions, leading to increased alveolar permeability to cause ARDS. Our current studies are aimed at investigating the genes differentially expressed, and signaling pathways modulated by the enhanced binding of galectin-3 to the de-sialylated receptors on the A549 cell surface during IAV infection, that lead to the observed enhanced expression of MMPs, disruption of airway epithelial tight junctions, and increase in cell-cell permeability. [Supported by grants IOS-1656720 (NSF) and R01GM070589 (NIH) to GRV; MI is supported by Fulbright grant (ID: PS00219036) and Ratcliff Environmental Entrepreneurial Fellowship (REEF)].

(P055) Marfan syndrome-related variation of the putative POGLUT2 and POGLUT3 consensus sequence results in abberant O-glycosylation of fibrillin-1

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Marfan syndrome (MFS) is a heritable autosomal dominant genetic disorder that affects ~1 in 7500 people. The disorder is characterized by severe connective tissue problems in the heart, lungs, and eyes. The underlying cause behind this disorder is variant forms of the protein fibrillin-1 (FBN1), a major component of the extracellular matrix (ECM). FBN1, along with elastin, forms elastic fibers within the heart and lungs, while non-elastic fibers are found in the eyes. Recently, it was shown that FBN1 is a major target of the novel Protein *O*-GLUcosyltransferases, POGLUT2 and POGLUT3. These enzymes mediate the addition of an *O*-glucose modification to Epidermal Growth Factor-like repeats (EGFs), small cysteine-rich protein modules that constitute the majority of FBN1. Within these EGFs is a putative consensus sequence, C^3-X-N-T-X-G-**S**-F/Y-X-C^4, where C^3 and C^4 are the third and fourth conserved cysteines of the EGF, 'X' denotes any amino acid, and the bolded '**S**' indicates the serine that is specifically modified by these enzymes. FBN1 consists of 47 EGF repeats, with ~60% of them modified at high stoichiometry by POGLUT2 & 3. Currently, the function of the *O*-glucose modification is unknown, as is its status in the context of Marfan syndrome. Utilizing PCR mediated mutagenesis, we introduced amino acid variants to a FBN1 N-terminal plasmid construct (FBN1-N, containing EGFs 1-26), replicating variants observed in MFS patients. These variants occurred within the putative consensus sequence modified by POGLUT2 & 3. These constructs were expressed in WT HEK293T cells, which endogenously express POGLUT2 & 3, allowing us to compare relative glycosylation levels of variant sites to WT sites. Some variants presented no change in *O*-glucosylation, while others significantly reduced glycosylation levels or produced entirely new glycoforms when compared to WT modification sites. Variation of the modifiable 'S' position eliminated glycosylation as expected. Varying the 'T' position of the consensus had a large effect, significantly reducing glycosylation. In one EGF, variation of the 'N' position produced elongation of the *O*-glucose with xylose at the affected site, which has not been observed in any WT sites. Changes in glycosylation were restricted to the EGF that contained the variation; glycosylation of other EGFs within FBN1-N were unchanged. Preliminary secretion assay data suggests that loss of glycosylation in one EGF results in impaired secretion of the entire FBN1-N construct from WT HEK293T cells. Further characterization of secretion levels, protein stability, and protein-protein interactions of both WT and variant protein will provide insight towards the impact of the *O*-glucose modification on FBN1 function. This work was supported by NIH GM061126 and HL161094. NRK and DBW were partially supported by T32 GM107004.

(P056) Determination of Isokinetic Ratios for MUC1-Derived Synthetic Glycopeptide Combinatorial Libraries

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Mucin 1 (MUC1) is a highly glycosylated transmembrane protein, which has emerged as a major target for the development of cancer therapeutics and vaccines. Approaches to MUC1-based immunotherapy rely on exploiting the overexpression and aberrant glycosylation of MUC1 in multiple cancer types. The isolation of well-defined tumor associated MUC1 from natural

sources has proven to be impossible due to the natural epitope heterogeneity found on the surface of cancer cells. Recent efforts have been focused on the fully synthetic MUC1-based cancer vaccine. Our approach involves synthesis of a diverse library of aberrantly glycosylated tumor associated 20-mer MUC1 peptides, harboring five possible glycosylation sites (Thr^4, Ser^5, Thr^9, Ser^{15}, and Thr^{16}), as a tool to model the diversity in the type and positions of *O*-glycans on MUC1. The positional scanning approach was used for the synthesis of glycopeptide library with tumor associated cancer antigens attached to Thr and/or Ser at positions corresponding to the potential glycosylation sites by "tea bag" method. These Positional Scanning Synthetic Glycopeptide Combinatorial Libraries (PS-SGCLs) display native-like heterogeneous and aberrant epitope presentation as seen on the surface of cancer cells. A key step in the design and development of PS-SGCLs is determination of the isokinetic ratios, that ultimately yield equal molar mixtures of the MUC1 glycopeptides. A protocol was established for the simple mixture of two components, the pentafluorophenyl esters of glycosylated Ser/Thr and a non-glycosylated Ser/Thr, and then the same approach was applied to the three component mixtures containing Tn, sTn, or no glycan attached to the Ser/Thr side chain. Mixtures are incorporated in different ratios at randomized positions. The effect of neighboring glycosylation on isokinetic ratios was also evaluated. To increase the reaction rate, couplings were performed in the presence of 1-hydroxybenzotriazole (HOBt). The ratio of products formed was analyzed by RP-HPLC and compound identity was confirmed by MALDI-TOF. The isokinetic ratios for each of the five glycan positions were determined within the limits of experimental error. The optimal ratios for the *O*-glycosylated Tn and sTn building blocks were found to be similar. To simplify the sTn synthesis a more convenient on-resin removal of acetyl and methyl ester protecting groups from MUC1 glycopeptides was developed.

(P057) Deficient Protein Glycosylation and Compromised Functional Integrity of PMM2 Deficient Neurons and Brain Organoids

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Congenital Disorders of Glycosylation (CDG) are a group of more than 160 rare metabolic disorders that occur due to abnormal protein and lipid glycosylation. Phosphomannomutase 2 (PMM2) is essential for normal N-linked glycosylation. PMM2-CDG is the most common CDG and has an overall prevalence of 1:20,000. The most severe symptoms are the neurological presentations including developmental delay and seizures. How PMM2 deficiency affects neural structure and function however remains elusive, mostly due to the lack of proper *in vitro* neuronal model systems of PMM2-CDG. Leveraging induced pluripotent stem cell technology, we differentiated excitatory cortical neurons (iNeurons) and developed brain organoids from 3 individuals with PMM2-CDG (*PMM2*c.422G>A/c.647A>T, c.548T>C/c.422G>A, and c.422G>A/c.415G>A) and 3 age-matched controls. First, we examined the proliferation of neural progenitor cells (NPCs) by Sox2 and Nestin immunohistochemistry of 30-day old cortical brain organoids. We found no difference in proliferation between patients and controls brain organoids. Compared to controls, PMM2 deficient iNeurons displayed irregular network burst rates and long burst activity containing 3-8 microbursts. We observed more condensed and shorter network bursts and found increased variability in the interval between network bursts in PMM2-iNeurons vs. controls. We also performed comparative quantitative proteomics and glycoproteomics by LC-MS/MS in cortical brain organoids. Protein expression level in PMM2 brain organoids was comparable to controls. We found, however, a robust reduction in protein glycosylation in PMM2 brain organoids vs controls. Our studies in PMM2 neuronal model systems showed deficient protein glycosylation and early functional neuronal deficits that could be the primary drivers of increased susceptibility to neurological symptoms in individuals with PMM2-CDG.

(P058) Towards understanding why N-glycosylation pathways evolved differently in microalgae

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For the past few decades, microalgae have attracted attention as they are used for various industrial applications including biofuels, cosmetics, nutraceuticals and pharmaceuticals. Regarding the latest applications, microalgae such as *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, and *Dunaniella salina* have recently been used as cellular biofactories for the production of biologics such as antibodies, SARS-COV2 RBD or EPO. It is therefore essential to understand their N-glycosylation pathway as 75% of biopharmaceuticals are glycoproteins and it is well established that their N-glycan structures have an impact on their functionality, half-life and immunogenicity. Therefore, several works published in the last three years have been devoted to the detailed characterization of *N*-glycan structures synthesized by microalgae as well as to the functional characterization of the

glycoenzymes involved in the synthesis of these *N*-glycans in the ER and Golgi apparatus. The results obtained in these studies will be presented. They highlight the fact that *N*-glycosylation pathways in microalgae have evolved differently, opening many new scientific questions concerning the regulation of the *N*-glycosylation pathways as well as the specific physiological roles of *N*-glycans in microalgae. This work is financially supported by the French government through the ANR agency under the Grand Défi Bioproduction and Biologics (ANR-21-F2II-0005) and through the ANR PRCE DAGENTA project (ANR-21-CE20-0038-001).

(P059) Identifying and characterizing ganglioside-mimicking structures on gut commensals

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Campylobacter jejuni is the leading cause of bacterial foodborne illness in the United States. Many isolates of *C. jejuni* express variable lipooligosaccharides (LOS) that mimic human glycolipids including gangliosides commonly found on neurons. A subset of patients with *C. jejuni* enteritis generate antibodies against the ganglioside-mimicking LOS on the bacterium, which cross-react with gangliosides on nerve cells, resulting in the autoimmune paralysis known as Guillain-Barré Syndrome (GBS). Our lab has identified additional bacteria that express ganglioside-like structures from poultry. Unlike *C. jejuni* strains that express the mimic on their LOS, the new isolates attach the mimicking oligosaccharides as O-linkages on glycoproteins. We will present data demonstrating the workflow involved in identification of these new microbes and mass spectrometry data summarizing the modified glycoproteins. Future NMR studies will confirm the absolute structure of the O-linked glycan and test purified glycoproteins in immunological assays to determine how the innate immune system reacts to these mimics in the induction, or suppression, of autoimmunity.

(P060) Modularity of the hydrophobic core and evolution of functional diversity in fold A glycosyltransferases

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Hydrophobic cores are fundamental structural properties of proteins typically associated with protein folding and stability; however, how the hydrophobic core shapes protein evolution and function is poorly understood. Here, we investigated the role of conserved hydrophobic cores in fold-A glycosyltransferases (GT-As), a large superfamily of enzymes that catalyze formation of glycosidic linkages between diverse donor and acceptor substrates through distinct catalytic mechanisms (inverting *versus* retaining). Using hidden Markov models and protein structural alignments, we identify similarities in the phosphate-binding cassette (PBC) of GT-As and unrelated nucleotide-binding proteins, such as UDP-sugar pyrophosphorylases. We demonstrate that GT-As have diverged from other nucleotide-binding proteins through structural elaboration of the PBC and its unique hydrophobic tethering to the F-helix, which harbors the catalytic base (xED-Asp). While the hydrophobic tethering is conserved across diverse GT-A fold enzymes, some families, such as B3GNT2, display variations in tethering interactions and core packing. We evaluated the structural and functional impact of these core variations through experimental mutational analysis and molecular dynamics simulations and find that some of the core mutations (T336I in B3GNT2) increase catalytic efficiency by modulating the conformational occupancy of the catalytic base between "D-in" and acceptor-accessible "D-out" conformation. Taken together, our studies support a model of evolution in which the GT-A core evolved progressively through elaboration upon an ancient PBC found in diverse nucleotide-binding proteins, and malleability of this core provided the structural framework for evolving new catalytic and substrate-binding functions in extant GT-A fold enzymes.

(P061) Effect of bisection on the 3D structure and recognition of N-glycans during maturation with implications of the expression of MGAT3 in biological function

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N-glycosylation is a highly conserved post-translational modification in eukaryotic cells. N-glycosylation relies on highly coordinated, complex cellular machinery operating through the endoplasmic reticulum (ER) and the Golgi, ultimately translocating

the glycosylated protein through the secretory pathway. The diversity of N-glycans is cell-dependent and their heterogeneity relies heavily on the expression of linkage and/or monosaccharide-specific glycoside hydrolases (GHs) and glycoside transferases (GTs), responsible for branching and functionalisation. In this work we are interested in the modification carried out by the N-Acetylglucosaminyltransferase-III, also known as MGAT3 from the encoding gene, which is responsible for linking a GlcNAc in b4 of the central mannose on an N-glycan pentasaccharide core, also called bisecting GlcNAc. This modification has been reported by some research groups to inhibit the functionalization of the N-glycans arms and overexpression of MGAT3 has been found to be linked to cancer and only common in specific tissues, such as brain and kidney. Meanwhile there is irrefutable evidence of bisection occurring in the context of fully functionalized biantennary N-glycans. In this work, we explore the structural architecture of these bisected N-glycans comparatively with fully functionalised tri- and biantennary N-glycans using molecular dynamics (MD) simulations. We use this information to characterise their potential recognition by two GTs involved in the maturation of N-glycans, namely beta-1,4-Galactosyltransferase 1 (b4GAlT1) and alpha-(1,6)-fucosyltransferase (FUT8), to gain insight into 'if and how' bisection hinders the maturation of N-glycans with implications of the expression of MGAT3 with the N-glycans' glycocode.

(P062) Ganglioside interactome revealed with clickable photoaffinity bifunctional ganglioside probes

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Gangliosides, sialic acid-containing glycosphingolipids, are mostly found on cell surfaces with their ceramide lipids embedded in the membrane and their glycans extending out. They drive cell physiology and pathology, in part, by interacting with proteins in the same plasma membrane (cis) or with proteins from outside the cell (trans, in the extracellular milieu or on opposing cells). To identify these proteins - the ganglioside interactome - we synthesized bifunctional ganglioside probes, each derivatized with an alkyl diazirine for photoaffinity labeling and an alkyne for click chemistry. Tags were covalently added at different sites on different gangliosides to expand interactome coverage. A short bifunctional tag (TagA) was installed on ganglioside sialic acids (Sia-TagA), or on galactose residues (Gal-TagA) by selective glycan oxidation, reductive amination and coupling via amide linkage to TagA. In addition a long bifunctional tag (TagB) was installed on the ceramide of different gangliosides by enzymatically removing the ceramide fatty acid amide and replacing it with TagB via amide linkage. Chemically synthesized ganglioside probes were chemoenzymatically converted to create other tagged gangliosides and thereby expand the ganglioside structural repertoire. For example, GD1a-derived probes were synthesized by chemoenzymatic addition of α-2,3-linked sialic acid to the terminal galactose of GM1-derived probes. Using a combination of natural sourcing, chemical synthesis and chemoenzymatic conversion, bifunctional probes of GM3, GD3, GM1, GD1a, GD1b, and GT1b were prepared. Ganglioside probes were delivered to human cells in culture by conjugating them with methyl-β-cyclodextrin. Subsequent visualization on live cells by click chemistry installation of a fluorophore revealed that the probes were selectively delivered to cell surfaces. To identify the ganglioside interactome, the delivered probes were photo-crosslinked to adjacent proteins, the proteins extracted, click-biotinylated and enriched by neutravidin pull-down. Captured ganglioside-protein complexes were processed for proteomic mass spectrometry, both label-free and with isobaric mass tagging. Gene ontology analyses revealed that ganglioside-tagged and captured proteins were primarily from the cell surface, consistent with their site of delivery. These data indicate that minimally disruptive bifunctional ganglioside probes can be synthesized and used to study ganglioside-protein interactions on human cells. The probes, bifunctionally tagged at different sites on each ganglioside, may reveal the binding specificity and interaction sites of ganglioside-interacting proteins. Supp by NIH grant U01 CA241953 and T32 GM080189 (MJP).

(P063) Evidence for a Sequestration Mechanism for the Skp1 α-Galactosyltransferase Gat1 in Toxoplasma gondii

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Gat1 catalyzes the addition of the final galactose of the pentasaccharide found on Skp1 from the parasite *Toxoplasma gondii*. Skp1 is an adaptor of the Skp1-Cullin1-F-Box Protein (FBP) E3 ubiquitin ligase (SCF) complex whose glycosylation is primed by the oxygen-dependent prolyl hydroxylase PhyA. Substantial evidence Indicates that oxygen-dependent glycosylation promotes disassembly of the Skp1 homodimer and assembly with FBPs, which comprise a family of SCF substrate receptors and other

targets. Genetic analysis shows that deleting Gat1 in a wild-type background has a modest growth phenotype that is strongly exacerbated in a PhyA-KO background. This was surprising since Gat1's only known substrate is Skp1 with a tetrasaccharide whose generation depends on the prior action of PhyA. Co-immunoprecipitation data indicate that, unlike the other GTs, Gat1 stably associates with Skp1 *in vivo* leading us to hypothesize that Gat1 plays an additional role in associating with non-substrate glycoforms. Gel filtration and analytical ultracentrifugation (AUC) indicate that Gat1 directly and stably interacts with Skp1. Glycosylation is suggested to affect how many Skp1 molecules associate with the highly stable Gat1 dimer. This may be related to the dissociative effect of glycosylation on Skp1 homodimerization as observed using gel filtration and AUC. Building on the known structure of each protein, AlphaFold modeling suggests that the Gat1 binding interface on Skp1 overlaps with its homodimerization and FBP interfaces. This model is supported by evidence that Gat1 inhibits Skp1 hydroxylation and competes with FBP binding to Skp1 *in vitro*. These findings offer a biochemical mechanism for the genetic data by suggesting a second role for Gat1 in titrating the availability of Skp1 available to the SCF complex. A related effect was previously proposed for the unrelated terminal glycosyltransferase of *Dictyostelium* Skp1, suggesting that this mechanism was a driving force for convergent evolution.

(P064) Glycan detection based on Surface-enhanced Raman scattering and machine learning

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A simple, low-cost, and highly accessible biosensor to monitor the glycosylation state would benefit the glycobiology community. Here, we present a new glycan detection platform that integrates surface-enhanced Raman spectroscopy (SERS), boronic acid chemistry, and machine learning algorithms. SERS provides fingerprint information of molecules, serving as a great tool for glycan identification. Most importantly, SERS can distinguish isomeric structures, allowing for isomeric glycan detection. In addition, boronic acids were used to not only selectively capture glycans, but also serve as the reporter molecule due to their unique vibration modes. Boronic acids have different affinities toward different pairs of hydroxyl groups in glycan molecules. When a glycan binds with a boronic acid, the intramolecular hydrogen bonding and the charge distribution alter the vibration modes of the boronic acid molecule. Therefore, boronic acid could induce a unique SERS spectrum for each glycan structure, allowing the identification of glycan molecules.

While the binding between boronic acids and glycans resulted in unique Raman spectra, it is challenging to analyze those highly complex spectral patterns. Machine learning was applied to mitigate this issue, enabling more accurate and sensitive glycan identification. Furthermore, machine learning algorithm could provide a quick and straightforward way of visualizing the results without additional human inputs. By integrating SERS, boronic acid receptors, and machine learning, we were able to distinguish the stereoisomers, structural isomers, and saccharides with different linkages. Moreover, we successfully quantify sialic acid concentration in milk oligosaccharides with the femtomolar concentration range using a machine learning regression algorithm.

In summary, we demonstrated a new detection platform for glycan identification and quantification. The simple detection protocol will allow end users to frequently conduct the assay in their own laboratories without expensive equipment. Moreover, end users who do not have extensive knowledge of bioinformatics can perform data analysis using sophisticated machine learning software.

(P065) One-Pot MultiEnzyme (OPME) Sialylation Kits for Custom Ganglioside Synthesis

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Gangliosides are attractive commercial targets for biomedical research, diagnostics, and therapeutics due to their critical roles in pathogen infection, cell-cell communication, and inflammation. Neuroprotective and neurorestorative functions of gangliosides have been explored for treating Parkinson's disease and other neurological indications such as traumatic brain injury. Isolation of specific gangliosides from animal brains cannot sustainably meet therapeutic demand and raises risks of zoonotic contamination. Chemoenzymatic synthesis is an efficient alternative that minimizes protection and deprotection steps and avoids undesired regio- and stereoisomers. IMCS is commercializing kits that enable facile and affordable enzymatic synthesis of custom gangliosides from a synthetic lactosylsphingosine acceptor. Here we present our first two One-Pot MultiEnzyme (OPME) kits, which enable synthesis of GM3 and GD3 gangliosides. Each kit contains an inorganic pyrophosphatase, CMP-sialic acid synthetase, and one of two sialyltransferases, which were prepared at multi-gram scale via fed-batch fermentation, immobilized metal affinity chromatography, and size-exclusion chromatography. Liquid storage formulation and reaction optimization resulted

in robust OPME systems that rapidly produce Neu5Aca2–3-lactosylsphinogosine (GM3Sph) and Neu5Aca2–8-Neu5Aca2–3-lactosylsphinogosine (GD3Sph). Additional OPME kits that enable synthesis of GM2, GM1, and other gangliosides are in development. These OPME kits will allow the broader scientific community to access custom gangliosides to better understand their biological roles.

(P066) Application of a new poly-N-acetyllactosamine endo-β-galactosidase to tissue and biofluid characterization in multi-enzyme N-glycan MALDI-QTOF imaging mass spectrometry workflows

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Poly-N-acetyllactosamine ([GlcNAc β(1-3)Gal β(1-4)]; poly-LacNAc) extensions attached to glycoconjugates are consistently reported in multiple cancer types to be important mediators of tumor progression, immune recognition and immunotherapy responses. Fucosylated, sialylated and un-modified poly-LacNAc structures are routinely identified in human tumor tissues when evaluated for N-glycan distributions by MALDI imaging mass spectrometry. To further evaluate poly-LacNAc N-glycan structures in human samples, a newly characterized recombinant endo-β-galactosidase from *Flavobacterium keratolyticus* was evaluated for use in MALDI-QTOF imaging mass spectrometry (IMS) applications in combination with PNGase F and sialidases. The endo-β-galactosidase specifically cleaves internal β(1-4)-galactose linkages in unbranched, repeating poly-LacNAc structures, releasing LacNAc di-saccharides as products. For initial characterization, breast and kidney cancer tissues were selected that were previously shown by N-glycan IMS to have a high abundance of poly-LacNAc species. The tissues were pre-treated for digestion with the endo-β-galactosidase prior to spraying of PNGase F, and then compared to the original distributions of poly-LacNAc species. In breast cancer tissues, N-glycans with 1-5 poly-LacNAc extensions were shown to be significantly decreased after endo-β-galactosidase digestion. A corresponding increase in basic bi- and tri-antennary precursor N-glycans, reflecting loss of LacNAc groups, were also detected. There was also an unexpected increase in high mannose N-glycan species detected in the endo-β-galactosidase treated tissues, implying that removal of poly-LacNAcs could improve access of PNGaseF to smaller N-glycans in these tumor regions. Ongoing characterizations include the effect of co-digestion with sialidase, and other glycosidases, and in a broader panel of tumor tissues and cancer serum samples. It is expected that this novel LacNAc exoglycosidase could find broad utility in characterizing the many functions of poly-lactosamines in cancer and immune glycobiology.

(P067) Commercial Production of Recombinant Sialic Acid Aldolase for One-Pot Multi-Enzyme (OPME) Synthesis of Neu5Gc-Containing Sialosides

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The sialic acids Neu5Ac and Neu5Gc are common components of mammalian glycoproteins, glycolipids and milk oligosaccharides. Of the two, humans only synthesize Neu5Ac, but they can incorporate Neu5Gc from dietary sources such as red meats and dairy into tissues, eliciting an immune response which leads to inflammation and increased risk of carcinomas. The disease relevance of sialic acids and the biological importance of diverse sialoglycans make these molecules an important target for research, but can prove to be expensive to purchase, or difficult and time-consuming to make through chemical synthesis or fermentation. Adding further complexity are different types of linkages (a2-3, a2-6, a2-8) and a large diversity of underlying asialoglycans (Galβ, GalNAcβ, Lewis X, etc.) which modulate the biological impact of both Neu5Ac and Neu5Gc sialoglycans. Purified bacterial enzymes combined in a one-pot multi-enzyme (OPME) system are an efficient way to generate sialic acids and sialoglycans with specific desired linkages or complex structures. Our collaborators and other researchers have previously developed and characterized recombinant enzymes for sialic acid synthesis and sugar activation and numerous sialyltransferases and glycosyltransferases capable of generating a wide variety of complex structures. Here, we demonstrate multi-gram-scale production and purification of a bacterial sialic acid aldolase in *E. coli*, its stabilization via storage buffer formulation, and its application in OPME systems for efficient chemoenzymatic sialoglycan synthesis. All chemicals were purchased from MilliporeSigma, Carbosynth, ThermoFisher or Cayman Chemicals. Recombinant enzymes were expressed with polyhistidine tag and purified by IMAC on an Akta FPLC (Cytiva). Enzymes were assayed using malachite green and/or HPAEC-UV on a Vanquish UPLC (Thermo). Storage buffers were optimized based on enzyme melting temperature and aggregation temperature values determined by intrinsic fluorescence and light scattering measurements on an UNcle protein stability screening platform (Unchained Labs). Recombinant expression of the bacterial sialic acid aldolase by fed-batch fermentation improved expression yields to over 7 g/L. Storage buffer formulation improved the melting temperature of the enzyme in its storage buffer by 15°C.

By combining the aldolase with a CMP-sialic acid synthetase and a pyrophosphatase, we demonstrate the production of CMP-Neu5Ac and CMP-Neu5Gc. Using stereo- and regioselective bacterial sialyltransferases, we additionally demonstrate the OPME synthesis of sialosides bearing Neu5Ac and Neu5Gc using the substrate analog LacβMU. We expect that these enzymes, alongside others currently in development, will provide cost-effective means for the research community to synthesize a wide array of rare and complex sialoglycans.

(P068) Evaluating N-Glycome Changes in Metastatic Neuroendocrine Prostate Cancer Tissues and Patient-Derived Xenograft Models Using MALDI Imaging Mass Spectrometry

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Metastatic prostate cancer (PCa) most frequently spreads to the bones, accounting for 85-90% of cases, but it can also spread to distant lymph nodes, liver, thorax, brain, gastrointestinal tract, and kidneys. In previous studies (Nguyen et al., Prostate, 2017, 77, 654), a cohort of castration-resistant prostate tumor tissues derived from the liver, lymph nodes, bones, bladder, and fat were used to generate patient-derived xenograft (PDX) models in the LuCaP series. Tissue microarrays (TMA) derived from the LuCaP series were obtained, and represented the original metastatic tumor tissues, tumors from the first PDX generation, and tumors from additional generations. These TMAs were processed for N-glycan MALDI imaging mass spectrometry (IMS) analysis on a Bruker MALDI timsTOFfleX mass sprectrometer using established protocols. For comparison, a series of clinical samples from neuroendocrine prostate cancers were evaluated by N-glycan MALDI-IMS. spatial distribution throughout each tumor. For most tumor tissues, an average of 70-80 N-glycan species were detected, with a majority being branched multi-fucosylated core- and outer-arm structures. An N-glycan fucose score was developed based on the number of multi-fucosylated glycans detected in each sample, allowing comparison across the TMA cores and clinical tissues. It was concluded that metastatic tumors derived from the liver as well as clinical neuroendocrine tumors exhibited the most fucose-rich glycome and highest fucose scores. Primary prostate tumors and tumors derived from trans-urethral resectioning of the prostate were among the lowest fucose-scoring tumors, supporting a conclusion that more aggressive tumors exhibit a more richly fucosylated N-glycome. Increases in fucose scores were also observed for later generation LuCaP tumors serially passaged and selected for robust tumor growth. This workflow can be adapted to include additional preparation to target core fucosylated structures as well as a 2,3 or a 2,6 sialic acids for additional glycomic characterization of the tissues.

(P069) The Glycan Array Data Repository

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Glycan arrays have grown greatly over the past 2.5 decades regarding the diversity of the immobilized glycans they present on the slides for query of binding activities and the broad range of biomedical domains they have impacted. The rich datasets generated by various internationally developed array formats have provided new knowledge regarding the structural specificities of known glycan binding proteins and new discoveries of previously unexpected glycan binding activities. These binding activities, when integrated with knowledge of the tissue, cell, and disease-specific expression of the candidate binding proteins and their recognized glycan ligands, promise to further accelerate appreciation of glycan structure and function in disease and normal tissue. Although, a huge number of array datasets have been generated since the first glycan arrays became available around 2000, a resource that systematically archives glycan array data and associated metadata is lacking. A few groups or consortia, such as the CFG and the Glycosciences Laboratory (Imperial College London), provide their own data on their respective group webpages. However, these webpages do not allow for public submission of data and are limited to the data generated by the corresponding group. Furthermore, there has been no agreement on metadata or common data formats between these groups that would enhance the interoperability and FAIRness of the data. The Glycan Array Data Repository is a public database which allows submission of array data independent of the array format or investigator-specific features. The submission system supports multiple glycan array raw data formats and requests associated metadata in accordance with the glycan array minimum information guidelines developed by the MIRAGE initiative (https://www.beilstein-institut.de/en/projects/mirage/). Uploaded data and metadata are harmonized into common data formats and stored in the database together with the originally submitted files. Once data is released to the public, datasets can be browsed or downloaded from the repository webpages without the need of a user account. The Glycan Array Data Repository is support by NIH Glycoscience Common Fund (1U01GM125267-01).

(P070) Role of natural killer (NK) cells in glycosaminoglycan-mediated anti-cancer immunity

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In the majority of patients with colorectal cancer (CRC), the overall response rate to immune-modulating therapies has been found to be limited. This resistance to therapy is attributed to low tumor mutation burden, neoantigen expression, and the inability of T cells to kill HLA-I negative tumor cells. To overcome this, novel strategies are being directed towards Natural Killer (NK) cells. Advantageously, NK cells can preferentially target cancer stem cells (CSCs) via stress ligand recognition. We have previously shown that glycosaminoglycans (GAGs) of a specific chain length, and synthetic mimetics thereof, selectively inhibit colon CSCs both *in vitro* and *in vivo*. In the current study, we evaluated the immunomodulatory role of natural GAGs as well as synthetic GAG mimetics (SGMs) in regulating the anti-tumor immune response.

8-12 wk old C57bl/6 mice were peritoneally injected with either SGM or natural GAG (Fucoidan) and the corresponding vehicle for 2 wks. Splenocytes were harvested, expanded in IL-2 containing media with or without pulsing with respective GAG/SGMs, and subjected to co-culture with syngeneic MC38 colon cancer cells. The effects of each treatment on immune modulation was evaluated using a) multiplex flow cytometry - Cytek® immune profiling assay; and b) cytotoxicity in cancer cells using MTT assay. PDL-1 expression and CSC marker expression in GAG/SGM treated MC38 spheroids was analyzed using western blotting and qPCR. *In vitro* MACS enriched human peripheral blood NK cells were stimulated *ex vivo* with GAG/NSGMs and analyzed for activation using flow cytometry.

Unbiased multiplex flow cytometry revealed a robust activation of NK cells from SGM-treated splenocytes co-cultured with tumor cells evident by increased expression of CD335 (NKp46) activation receptor. The MC38 colon cancer cells co-cultured with splenocytes from SGM-treated animals and pulsed with SGM *ex vivo* showed increased tumor cell cytotoxicity compared to splenocytes from vehicle controls. Furthermore, our preliminary findings showed that human peripheral blood NK cells ($CD45^+CD3^-CD56^+$) showed activation of NK cells (CD69 expression) with both GAGs and SGMs but showed a meaningful proliferative response ($Ki67^+$) upon *ex vivo* stimulation with GAG oligosaccharide HS06 (Heparin Hexasaccharide) and not polymeric GAG HS36. Mechanistically, a significant reduction in Pdl-1 protein expression was observed in MC38 spheroids concurrent with downregulation of various CSCs maker genes, e.g., Oct4, Sox2, and c-my when treated with various SGMs *in vitro*. We also observed downregulation of Pd-1 and Cxcr4 on the T-cells co-cultured with MC38 tumor cells following treatment with an SGM compared to vehicle controls.

The work highlights the role of novel SGMs in the activation of NK cells, that can preferentially target CSCs. These findings are highly significant with much therapeutic implications, especially in the context of tumor cell recognition and elimination by immune cells.

(P071) α2,6 sialylation, but not α2,3 sialylation, of the N-glycans on TNFR1 modulates TNFR1 signaling to promote cell survival

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TNFR1 is a crucial cell surface receptor that regulates the balance between cell survival and cell death. TNFR1 is activated by TNFα, which is mainly produced by immune cells such as macrophages. Upon TNFα binding, TNFR1 first activates NFκB-mediated survival signaling, however, TNFR1 subsequently internalizes and then activates apoptosis. It is well-known that inhibiting TNFR1 internalization diverts signaling toward survival. In prior studies, we reported that the α2,6 sialylation of TNFR1 by the ST6GAL1 sialyltransferase hinders TNFR1 internalization, leading to increased NFκB activation and a block in apoptosis. In the current study, we addressed the critical question of whether the survival-promoting effects of TNFR1 sialylation are specific to the α2,6 sialic acid linkage. To this end, we used HEK293 cells harboring CRISPR-Cas9 mediated deletions in select sialyltransferases. Four HEK 293 cell lines were utilized: (1) Wild type (WT); (2) cells that lack α2,3 sialylated N-glycans due to the deletion of ST3GAL3, 4 and 6 (ΔST3); (3) cells that lack α2,6 sialylated N-glycans due to the deletion of ST6GAL1 and 2 (ΔST6); and (4) cells with re-expression of ST6GAL1 in the ΔST6 line (ΔST6-R). Differential sialylation of TNFR1 was confirmed by SNA lectin pulldown experiments. Cells were treated with TNFα and it was found that ΔST6, but not ΔST3, cells had markedly greater activation of apoptotic caspases than WT cells. Moreover, ΔST6 cells had reduced activation of pro-survival molecules including NFκB and AKT. Importantly, the phenotypic effects of ST6GAL1/2 deletion were completely reversed by re-expression of ST6GAL1, evidenced by the comparable behavior of WT and ΔST6-R cells. We then examined the effects of sialylation on TNFR1 internalization. After activation, TNFR1 is cleaved by the TACE enzyme and internalized via a clathrin-dependent mechanism. TNFR1 undergoes cleavage by TACE for internalization and apoptosis. Notably, ΔST6, but not ΔST3, cells displayed increased levels of a TACE-mediated cleavage product, suggesting TNFR1 is more readily cleaved and internalized in the absence of α2,6 sialylation. We next treated cells with inhibitors of TACE or dynamin to block cleavage and internalization, respectively. Blocking these steps eliminated the pro-apoptotic phenotype of ΔST6 cells. Finally, we analyzed

the effects of sialylation on the Fas death receptor, in light of our prior studies indicating that ST6GAL1 has similar effects on Fas and TNFR1. We determined that α2,6, but not α2,3, sialylation blocks Fas-mediated apoptosis. Taken together, our results suggest that ST6GAL1 plays a unique role in promoting cell survival that is not replicated by other sialyltransferases. Given the high levels of ST6GAL1 expression in cancer cells and some stem/progenitor populations, we posit that ST6GAL1 functions to protect cells from a cytotoxic inflammatory microenvironment, and also shield malignant cells from anti-tumor immunity.

(P072) Liquid Formulation Optimization for Stability and Activity Retention of Bacterial Enzymes

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Enzymatic cascades called one-pot multienzyme (OPME) reaction systems enable efficient synthesis of otherwise-challenging carbohydrate targets, and IMCS is commercializing several OPME systems to facilitate its adoption by non-specialists. Storage stability is an important part of any enzyme commercialization effort, but it is particularly important for OPME kits which require carefully determined ratios of enzymes. Here we present our progress in developing stable storage conditions for several recombinant enzymes using intrinsic fluorescence to screen protein stability, accelerated aging studies, and six-month stability studies. Liquid formulations were developed for seven bacterial enzymes, including an inorganic pyrophosphatase, CMP-sialic acid synthetase, sialic acid aldolase, and four sialyltransferases. These enzymes displayed melting temperature and aggregation temperature improvements of 10-20°C after three rounds of formulation screening. Accelerated aging studies and K_D/B_{22} measurements were used to make final determinations between lead formulations. Two enzymes have successfully completed six-month stability studies at -20°C, 4°C, and room temperature. These results demonstrate the value of liquid formulation optimization and the stability of IMCS's glycoenzyme products.

(P073) Structural insights on oligosaccharides in commercial infant formula products using ion chromatography-mass spectrometry (IC-MS)

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Interest in functional oligosaccharides has grown substantially because of their profound impact on the gut microbiome. As prebiotics, the health benefits of functional oligosaccharides often lead to their supplementation in infant formulas. The structural features of oligosaccharides are closely associated with their health benefits; and therefore, they need to be characterized in detail. We present a novel tool combining high performance anion chromatography (HPAE) and high-resolution MS for derivatization-free, high-resolution characterization of functional oligosaccharides in several infant formula products. Importantly, this strategy features easy sample preparation, premium isomer separations, and high-quality MS data for structural elucidation of oligosaccharides; thus, positioning it as a beneficial tool for the qualitative assessment of formula products and other products supplemented with prebiotics. Powdered formula products were prepared following manufacturer instructions. Samples were prepared to remove protein and isolate oligosaccharides, which were analyzed using a CarboPac PA300-4μm analytical column in a Dionex ICS-6000 system outfitted with an electrochemical detector and a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer. Prior to MS injection, the column effluent was passed through an electrolytically regenerated desalter. IC-MS was used for the analysis of both dairy- and soy-based formulas fortified with different functional oligosaccharides. A variety of oligosaccharides were characterized, including human milk oligosaccharides and other common prebiotic oligosaccharides such as galactose-, fructose-, and malto-oligosaccharides. High resolution separation of chemically similar but structurally heterogenous oligosaccharides is crucial for downstream mass spectrometric characterization. This method takes advantage of HPAE to revolve heterogeneous mixtures of oligosaccharides, including isomeric structures. Using this method, we confidently identified a diverse mixture of oligosaccharides, including synthetic 2'-fucosyllactose, milk-based sialyllactoses, and other functional oligosaccharides with degrees of polymerization up to 8 monosaccharide units. Structural isomers were well resolved by the anion-exchange column. Negative electrospray ionization and subsequent high mass accuracy Orbitrap analysis facilitated both the sequence and linkage characterization of functional oligosaccharides. Understanding the structural complexities in functional oligosaccharide spectral profiles is crucial, as many structural features are deterministic of their biological function as prebiotics. The important structural features therefore need to be characterized to ensure the final products meet the specifications. The unique workflow presented here provides confirmatory, orthogonal information for food oligosaccharides analysis, allowing for monitoring the product quality and identifying new peaks for quality control purposes.

(P074) Influence of Fringe Glycosyltransferases on Osteoclastogenesis

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Osteoclasts are multinucleated bone-resorbing cells with roles in both physiological and pathological skeletal remodeling. Osteoclastogenesis, the differentiation of osteoclasts from their monocyte/macrophage lineage precursors, is dependent upon RANK signaling, but other pathways, such as Notch, can influence their size, activity, and lifespan. As Notch signaling is modulated by selective glycosylation by the Fringe family of *O*-fucose-specific β1,3-N-acetylglucosaminyltransferases (GnTs), we sought to determine whether overexpression of individual Fringe glycosyltransferases alters osteoclastogenesis. We cloned full-length coding sequences for LFNG, MFNG, and RFNG from a mouse bone marrow cDNA library and inserted them into pMXs-Puro, a Moloney Murine Leukemia Virus (MMLV)-based gammaretroviral vector, and retroviral particles were generated via transient co-transfection with a VSV-G envelope plasmid into Platinum-E packaging cells. Following infection of RAW264.7 cells, which are immortalized macrophages/osteoclast precursors, we confirmed overexpression via RT-PCR, and subjected variable densities of parental and overexpression lines to RANKL-stimulated osteoclastogenesis. At the conclusion of the differentiation, we stained cells for Tartrate-Resistant Acid Phosphatase (TRAP) activity and assessed resultant morphologies. We found that LFNG-overexpressing (OE) cells produced similar osteoclasts to parental, but these osteoclasts appeared to have a longer lifespan as fewer LFNG-OE demonstrated fewer morphological indications of apoptosis. MFNG-OE cells demonstrated more extensive differentiation than parental, but showed a greater level of apoptosis at higher densities. RFNG-OE cells demonstrated reduced osteoclastogenesis with fewer and smaller osteoclasts and more cells demonstrating a macrophage-like morphology. These data suggest that different Fringe glycosyltransferases regulate different aspects of osteoclast activity with, potentially, LFNG promoting longer lifespans, MFNG promoting faster differentiation (and, thus, shorter lifespans), and RFNG suppressing differentiation. Future work will investigate the mechanisms underlying these differential effects through gene knockout.

(P075) Targeted therapeutic agent for Streptococcus pneumoniae stereotype 3 infection

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Streptococcus pneumoniae (Spn) is a Gram-positive bacterial pathogen, which has been a major threat to human health. Spn is the causal agent to the invasive pneumococcal diseases (IPD) and according to CDC, IPD causes 150,000 hospitalizations every year in the United States alone. Additionally, pneumococcal co-infections are a driving force behind the mortality associated with other infectious diseases, such as flu and more recent pandemic outbreak COVID-19. Importantly recent report from WHO confirmed that, Spn is an antimicrobial resistance (AMR) bacterial pathogen, and it is becoming resistant to antibiotic therapies which are widely used to treat Spn infections. Among more than hundred different Spn strains, serotype 3 (Spn3) has unique biological and physical structural challenges such as thick capsular polysaccharide (CPS), high viscosity and mucoid nature, which help Spn3 to escape from host immune responses. The CPS of Spn3 also renders it a highly unresponsive stereotype to the multivalent conjugate vaccine developed against Spn, and thus incidence rates of serotype 3 continue to raise. Because of the unresponsiveness to available pneumococcal conjugate vaccines (PCV) and antibiotics resistance, a novel therapeutic agent is necessary to control the invasive pneumococcal serotype 3 infection.

We have identified and cloned the *Pn3Pase* gene expressed by a *Paenibacillus* strain. The purified Pn3Pase enzyme degrades the CPS of the Spn3, rendering the bacterium immune-susceptible. We confirmed that recombinantly purified Pn3Pase can degrade the capsule of living Spn3; the unencapsulated Spn3 are not infectious. Also, Spn3 that is unencapsulated by Pn3Pase is efficiently targeted by phagocytosis which also limits the nasopharyngeal colonization in a mouse colonization model. We have also observed that Pn3Pase protects the mice from lethal challenge with Spn3. Pn3Pase is not toxic and repeated administration in mice does not stimulate significant host immune responses against the enzyme. Our results indicate that Pn3Pase serves as an alternative to antibiotics and could effectively make up for vaccine shortcomings, therefore a promising therapeutic agent against Spn3 infections.

(P076) Exploring the Role of MUC-Type O-Glycosylation in Alzheimer's Disease

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Alzheimer's disease (AD) is one of the most common neurodegenerative disorders linked to aging. It has a profound effect on the economy, healthcare system, and the society, and is projected to increase even further as the population ages. New evidence

continues to emerge to support the idea that deficiencies in APP trafficking and clearance of Aβ peptides is the initiating event of AD pathogenic processes. Efforts to understand the role of proteolytic cleavage of the amyloid-β precursor protein (APP) by α-, β-, and γ-secretases into the toxic amyloidogenic pathway have sparked interest in the role of MUC-type *O*-glycosylation in production and clearance of Aβ peptides. To date, the stimulating and inhibiting effects of glycosylation on enzyme activity have been reported. Thus, a better understanding of the role of *O*-glycosylation in the initiating events of AD pathogenic processes is necessary. With this goal in mind, we have synthesized native and Swedish-mutated ($Lys^{670}Asn/Met^{671}Leu$) (glyco)peptides with *O*-GalNAc moiety on Tyr^{681}, Thr^{663} and/or Ser^{667} to explore the role of glycosylation on conformation, secretase activity, aggregation kinetics of Aβ40, and cell toxicity. The chosen peptide sequences incorporate the β-secretase (BACE-1) ($M^{671}\sim D^{672}$ or $L^{671}\sim D^{672}$) and/or α-secretase (ADAM-10) ($K^{687}\sim L^{688}$) cleavage sites, located near and within the Aβ40 domain, respectively. CD analysis was carried out in four solvent systems to evaluate peptide environment and *O*-glycosylation induced conformational changes. Atomic force microscopy was used to image the morphology of the Aβ40 aggregates formed without or in the presence of APP (glyco)peptides and cell toxicity was studied in an AD culture model. The Swedish mutation and *O*-glycosylation were the key factors driving conformational changes. Furthermore, the level of β-secretase activity significantly increases for the glycopeptides containing the Swedish mutation compared to their nonglycosylated and native counterparts. Lastly, the glycopeptides impact the kinetics of Aβ40 aggregation by significantly increasing the lag phase and delaying aggregation onset, however, this effect is less pronounced for its Swedish-mutated counterparts.

(P077) Prolonged intermittent hypoxia causes reductions in protein synthesis and sarcopenia associated with upregulation of the Hexosamine Biosynthesis Pathway in models of COPD

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Chronic obstructive pulmonary disease (COPD) is the 4th major cause of death in the United States. Sarcopenia or skeletal muscle loss is a frequent complication in COPD that contributes to adverse clinical outcomes including mortality. As the disease progresses, worsening airway obstruction predisposes to nocturnal hypoxemia in a significant proportion of patients in a condition known as prolonged intermittent hypoxia (PIH). We hypothesize that PIH upregulates glycolysis and the Hexosamine Biosynthetic Pathway (HBP), a branch of glycolysis responsible for protein glycosylation, to cause sarcopenia or skeletal muscle loss.

Our *in vitro* model of PIH exposes differentiated murine C2C12 to 16 hrs normoxia followed by 8 hrs of hypoxia [1% oxygen] for 3 days to determine sarcopenic responses. Data were compared from myotubes either untreated or exposed to chronic hypoxia (3 days). In preliminary studies, PIH causes a sarcopenic phenotype in our *in vitro* murine myotube model as demonstrated by reductions in myotube diameter and protein synthesis (i.e. decreased mTORC1 signaling and puromycin incorporation). This was accompanied by increased expression of hypoxia-inducible factor 1-α (HIF1-α), reductions in TCA intermediates, mitochondrial oxidative dysfunction, and metabolic shift to glycolysis associated with upregulated glycolytic proteins, including upregulated enzymes and metabolites in the HBP. We have now validated our data *in vivo* with C57BL/6 wild type mice exposed to PIH (12 hrs normoxia/12 hrs hypoxia [10% oxygen]) also demonstrating upregulation of the HBP and a sarcopenic phenotype.

To determine whether upregulation of the HBP causes sarcopenia in response to PIH, we knocked out GFAT, the enzyme which catalyzes the first and rate-limiting step of the HBP. Our results showed significant reductions in PIH-induced global O-GlcNAcylation of proteins in GFAT KO myotubes indicating reduced HBP activity. Interestingly, upregulated protein synthesis via the mTORC1 signaling pathway was most pronounced due to PIH in GFAT KO myotubes. This was accompanied by increased myotube diameter and attenuation of a sarcopenic phenotype as compared to WT PIH myotubes. These findings are translationally relevant due to the perturbations in the HBP that have been demonstrated in human skeletal muscle from patients with COPD who experience prolonged intermittent hypoxia. Future studies focusing on the HBP are needed to identify mechanistic and therapeutic targets to reverse sarcopenia in COPD and other chronic diseases.

(P078) Spatial Profiling of Altered N-linked Glycosylation Patterns in Ewing Sarcoma Tumors by MALDI- Mass Spectrometry Imaging

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Ewing sarcoma (ES) is a rare pediatric cancer of the bone and soft tissues affecting adolescents and young adults, with peak incidence from ages 10 to 15 years. A clinical feature of ES is the accumulation of Periodic acid-Schiff positive (PAS+) aggregates.

PAS staining can detect different classes of oligosaccharides such as glycogen and N-linked glycans. While increased glycogen is known in ES, it is unclear whether N-linked glycan metabolism is dysregulated during ES progression. Herein, we report the N-linked glycan profile ES patient tumors of the bladder, tibia, chest wall, shoulder, testis, and rib compared to normal bone. We employed matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) and performed glycomics analysis. In summary, we found significant N-glycan dysregulation in the primary tumor versus normal control tissue. Tumor tissue has markedly increased high-mannose class of N-glycans, and a number of unique sialylated and bisecting glycans are altered in primary tumor tissue. These results suggest fundamental changes in glycan metabolism during ES tumor progression. This case report provides a unique insight into the N-linked glycan spatial and metabolic profile of ES patient sample can be expanded into future digital pathology and biomarker analyses.

(P079) Loss of O-glycosylation via neuronal Galnt2 knock-out in mice recapitulates GALNT2-CDG patient seizure phenotype

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Congenital disorders of glycosylation (CDG) are a group of neurogenetic disorders that disrupt cellular glycosylation machinery and exhibit multi-system dysfunction, including severe neurological deficits. These disorders emphasize that glycosylation is an essential posttranslational modification, yet the pathophysiology of neurological dysfunction in CDG remains unclear. Most CDG disrupt N-glycosylation, however, eight patients from five families with biallelic loss-of-function mutations in GALNT2, which encodes a Golgi-localized glycosyltransferase that initiates mucin-type O-glycosylation, confirms O-glycosylation is alsocritical to neurologic function. GALNT2-CDG patients exhibit global developmental delay,epilepsy, autistic features, and white matter changes on brain MRI.

A mouse model of the neurological aspects of this neurogenetic disorder, GALNT2-CDG, using a floxed *Galnt2* allele and cell-type specific Cre drivers, exhibits spontaneous seizures and deficits across numerous behavioral and learning domains. Time-locked video EEG recordings exhibit frequent abnormal electrographic spikes and identify seizures in the majority of neuronal KO mice, which increase in prevalence as the mice age. Power analysis of background EEG frequency composition demonstrates abnormalities across the major EEG frequency bands (delta 0.1–4.0 Hz, theta 4–8 Hz, alpha 8–13 Hz, beta 13–25 Hz, and gamma 25–50 Hz). Glycoproteomic analysis of synaptosomes isolated from brain identify candidate glycoproteins and disrupted O-glycosites that likely underly these abnormalities. Genetic dissection of the circuit suggests that molecular events in both excitatory and inhibitory neurons contribute to development of spontaneous seizures. These findings demonstrate the key role of O-glycosylation in neurons initiated by just one of 20 related enzymes and implicate a role of O-glycosylation in diverse neurological processes, including learning, memory, and neurotransmission.

(P080) Interaction of Multivalent and Non-multivalent Ligands with the Cation-independent Mannose 6-phosphate Receptor Measured by Surface Plasmon Resonance

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The 300 kDa cation-independent mannose 6-phosphate receptor (CI-MPR) functions in the biogenesis of lysosomes by trafficking newly synthesized lysosomal enzymes with mannose 6-phosphate (Man-6-P)-containing N-glycans from the trans-Golgi network to late endosomes. CI-MPR also binds several non-lysosomal proteins at the cell surface, such as the 7.5 kDa peptide hormone, insulin-like growth factor 2 (IGF2), and plasminogen. This multifunctional receptor's ability to interact with different ligands in a glycan-dependent (lysosomal enzymes) or glycan-independent (IGF2) process is made possible by its extracellular region, composed of 15 contiguous domains. Domains 3, 5, 9, and 15 bind N-glycans modified with Man-6-P, whereas IGF2's interaction maps predominantly to domain 11. Previous studies using X-ray crystallography, SAXS, hydroxyl radical protein footprinting, single-particle/negative-stain EM, and cryo-EM support that CI-MPR undergoes significant conformational changes as a consequence of pH as well as ligand binding. Although CI-MPR contains binding sites for different ligands, how a particular ligand affects the receptor's conformation or the ability of CI-MPR to interact with other ligands is poorly understood. To further evaluate this allosterically regulated receptor, we examined the ligand binding capabilities of CI-MPR by performing surface plasmon resonance (SPR) measurements on the endogenous receptor isolated from bovine serum. CI-MPR exists as a soluble protein in mammalian serum (sCI-MPR) and is released from the plasma membrane by matrix-metalloproteinases. We isolated sCI-MPR from newborn calf serum by affinity chromatography and subjected the receptor to proteolysis and mass spectrometry analysis. Peptides covering the entire extracellular portion of the mature protein (amino acids T48 - K2306) were detected, indicating that cleavage occurs near the transmembrane domain (A2314). SPR studies on sCI-MPR were carried out using two multivalent, Man-6-P-containing lysosomal enzymes, acid α-glucosidase (GAA), and

palmitoyl-protein thioesterase-1 (PPT1), and the non-Man-6-P-containing hormone IGF2 that represents a monovalent ligand. In experiments in which sCI-MPR was pre-incubated with IGF2 prior to the complex being flowed over the GAA- or PPT1-immobilized sensor surface, IGF2, but not the closely related peptide IGF1, inhibits CI-MPR's ability to bind the lysosomal enzymes GAA and PPT1 in a concentration-dependent manner, with half-maximal inhibition occurring at ~8 nM IGF2. In a complementary approach, sCI-MPR was flowed over the GAA- or PPT1-immobilized sensor surface for 3 min, followed by a second injection of buffer containing increasing concentrations of IGF2. IGF2, but not IGF1, enhances the dissociation of both sCI-MPR-GAA and sCI-MPR-PPT1 complexes in a concentration-dependent manner. These results support IGF2 acting as an allosteric regulator of lysosomal enzyme binding by CI-MPR. (NIH R01DK042667 to NMD)

(P081) Fucose signatures in peripheral blood glycoproteins are associated with reduced clinical benefit of immune-checkpoint inhibitors in metastatic melanoma

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The clinical success of immune-checkpoint inhibition (ICI) in melanoma has confirmed the merit of therapeutic strategies that boost the immune system to counteract cancer, leading to a sea change in treatment approaches and patient outcomes. However, only about half of patients derive a long-lasting benefit. While elevated PD-L1 expression and tumor mutational burden correlate with the likelihood to benefit from ICI therapy in some indications, these biomarkers have shown poor predictive performance in metastatic melanoma. By applying InterVenn's glycoproteomics platform to pre-treatment plasma samples from metastatic melanoma patients receiving anti-PD-1/anti-CTLA-4 therapy, we previously identified a panel of biomarkers that differentiate patients likely to derive a benefit from those unlikely to benefit from ICI. A laboratory-developed test based on these findings, DAWN^{TM} IO Melanoma, was developed and has been recently introduced into the market.

Here, we report the results of our analysis of the glycosylation patterns detected in pretreatment plasma samples from 205 patients with metastatic melanoma who received ICI therapy. When comparing glycopeptide structures, we identified a fucosylation signature in N-linked glycoproteins that identified individuals unlikely to benefit from ICI therapy. To test the validity of this observation, we engineered site-specific glycosylation features that represent the average number of specific monosaccharides at a given site, weighted by glycopeptide occupancy. Of 52 fucose-dependent features across our full research assay, 12 were associated with benefit from ICI therapy based on univariate Cox regression analysis (FDR <0.05). Two features were ultimately retained in a repeated cross-validated LASSO-regularized Cox regression model on a training set consisting of 40% of the cohort, yielding a hazard ratio (HR) of 5.1 (p=3e-05). A validation set consisting of 30% of the cohort was used to tune model hyperparameters. When applied to the remaining 30% of the cohort, this tuned model resulted in a HR of 2.6 (p=3e-02), indicating that fucose-dependent features stratified patients in groups that differ in the likelihood of deriving benefit from ICI therapy, such that patients with a risk score exceeding the selected threshold were nearly three times less likely to respond. We are currently investigating the fine structure, the drivers, and the roles of the observed N-glycosylation patterns with a battery of biochemical and cellular assays. Fucosylation of specific glycoproteins may impact ICI efficacy by modulating signaling pathways involved in T cell function and distribution within the tumor, as well as by affecting the metastatic potential of melanoma cancer cells.

(P082) Roles of POFUT1 and POGLUT1 for the effective transport of DLK1 to the cell surface

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Protein delta homolog 1 (DLK1) is a non-canonical ligand for the NOTCH signaling pathway. There are six epidermal growth factor (EGF)-like repeats in the extracellular domain of DLK1. EGF repeats are modified with *O*-glycans such as *O*-fucose, *O*-glucose, and O-GlcNAc. Herein we investigated the presence of *O*-glycans on human DLK1 in HEK293T cells by mass spectrometric analysis. Consistent with previous reports, *O*-fucose and *O*-glucose glycans were detected on EGF4 and EGF6. To explore the molecular roles of these *O*-glycans, we examined the turnover of newly synthesized DLK1 in HEK293 cells under the deficiency of glycosyltransferases responsible for transferring *O*-fucose, *O*-glucose, or *O*-GlcNAc glycans (i.e., POFUT1, POGLUT1, or EOGT). Loss of POFUT1 or POGLUT1 extended the turnover of DLK1. Next, we established a transport assay to the cell surface from the ER where DLK1 was released by the addition of biotin after accumulation through biotin-streptavidin interaction based on the retention using a selective hook system. The *O*-glycosylation of the reporter DLK1 is completed before the initiation of the transport, which is revealed by the *O*-glycan structures of the reporter DLK1 that were in accordant with the presence or absence of each *O*-glycosyltransferase. The transport assay showed that either loss of POFUT1 or POGLUT1

delayed the reporter DLK1 to the cell surface compared with wild-type cells. These data collectively suggest that POFUT1 and POGLUT1 are important for the effective transport of DLK1 from the ER to the cell surface.

(P083) Comparative analysis of N-glycan profiles of 15 organ tissues and tumors using MALDI imaging mass spectrometry

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Glycoproteins comprise approximately 80% of cell-surface proteins, serving in a variety of roles in both normal cells and tumorigenesis. N-glycans have many cellular functions including protein folding, protein localization, cell-cell signaling, and immune recognition. Different organs and tissue types display different glycan profiles, and these change in tissue-specific ways when cells become cancerous. However, no overview of normal and cancerous N-glycomes exists at a comparative level, as most studies focus on single tissue types and cancers. In order to study a broad range of human tissue glycomes using MALDI imaging mass spectrometry (IMS), we have generated two custom formalin-fixed paraffin-embedded (FFPE) tissue microarrays (TMAs) comprised of eighteen different tissue types – fifteen of which have at least three samples (bladder, breast, cervix, colon, esophagus, gastric, kidney, liver, lung, skin, pancreas, prostate, sarcoma, thyroid, uterus). These contain tissue cores from both normal and tumor regions for each sample, selected by a pathologist, allowing more in depth comparisons of the N-glycome differences between tumor and normal in multiple tissue types. Using established MALDI-QTOF IMS workflows and existing N-glycan databases, we were able to spatially profile the N-glycans present in each tissue core. A multi-enzymatic approach allowed for further information on structural composition, using EndoF3 to confirm core fucose structures and stabilization chemistry to distinguish sialic acid linkages. From a selected peak list of 104 N-glycans present in both TMAs, peak intensities for each glycan in each tissue core were used for statistical comparisons. Differences in the presence and abundance of structural features like high mannose/paucimannose, branching complexity, bisecting N-acetylglucosamine, fucosylation and sialylation were evaluated. In all samples, the two most abundant glycans detected were Hex5dHex1HexNAc4 and Hex5HexNAc4, and their singly sialylated forms were the two most common sialylated glycans detected. In many tissues, high mannose N-glycans were upregulated in cancers, as were highly branched N-glycans. Additionally, we have identified N-glycans significantly upregulated and/or downregulated in cancer for each organ. Use of endoF3 indicated that the majority of all singly fucosylated, bi-antennary N-glycans were core fucosylated in both normal and tumor tissues. In tumors, there are progressive increases in outer arm fucosylated structures in tri- and tetra-antennary N-glycan species. This study will provide structural information on a wide scale for both normal tissue types and cancers in these 15 organs. Because clinical archived FFPE tissues are used, the analyses can be readily expanded to other organs and tissue types. The goal is to generate an N-glycome database of normal and tumor compositions in each organ, providing a reference for future studies and diagnostic use of glycan IMS.

(P084) Breast milk antibodies to microbial glycoconjugates

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Human breast milk is a unique mix of nutrients and protective factors, which include complex oligosaccharides, cytokines, and immunoglobulins. Those factors have either prebiotic effects or directly contribute to the infant's immune defence. Secretory IgA make about 90% of all immunoglobulins in human breast milk. These antibodies are produced by plasma cells that migrate to the mammary gland from the gastrointestinal tract. The composition of the gut microbiota therefore influences the repertoire of breast milk antibodies and hence the range of protection conveyed to breastfed infants. The dense glycosylation of bacterial surfaces provides a vast diversity of carbohydrate antigens stimulating the production of carbohydrate-specific antibodies. Despite the ubiquity of glycosylation of the gut microbiota, much remains unclear about the specificity and cross-reactivity of antibodies recognizing bacterial glycan antigens. To characterize the specificity of breast milk antibodies towards microbial glycoconjugates, we have developed a microarray representing 102 distinct lipopolysaccharide (LPS) structures. The analysis of breast milk IgA, IgG and IgM on this microarray showed that antibody mainly react with O-antigens, given that rough LPS mutant lacking parts of their glycan moiety are poorly recognized. This analysis also pointed to a strong variability in the range of LPS antigens recognized between individual breast milk samples. The investigation of breast milk samples collected from

different geographical locations also pointed to significant differences in the types of LPS recognized by breast milk antibodies. Several LPS recognized by antibodies share carbohydrate epitopes with microbial pathogens prevalent in the regions, in which the breast milk samples were obtained. Therefore, LPS arrays may be used to identify O-antigens presented by commensal gut bacteria, which may provide cross-protection against common infectious diseases.

(P085) Synthesis of MUC1 peptide backbone bearing Tn and sialyl-Tn antigen for structural and functional studies with endogenous lectins

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Mucin 1 (MUC1) is a heavily glycosylated transmembrane protein, altered in both expression and glycosylation pattern in human carcinomas of the epithelium. The presence of incomplete or truncated glycan structures, often capped by sialic acid, commonly known as tumor-associated carbohydrate antigens (TACAs), on the cell surface is a well-known cancer biomarker and therapeutic target for different types of cancer. Accumulating evidence suggests that TACAs expression is associated with tumor escape from immune defenses through interaction with endogenous carbohydrate binding proteins (lectins). These interactions frequently result in the development of a protumor microenvironment, favoring tumor initiation, progression, metastasis, and immune evasion. Macrophage galactose binding lectin (MGL) is a C-type lectin receptor found on antigen-presenting cells (APCs) which facilitates the uptake of carbohydrate antigens for antigen presentation, modulating the immune response homeostasis, autoimmunity, and cancer. Considering the crucial role of tumor-associated forms of MUC1 and MGL play in tumor immunology, a thorough understanding of this interaction is essential for it to be exploited for cancer vaccine strategies. The specific goal of this research is to synthesize structurally well-defined chemical probes, mono and multiple glycosylated MUC1 glycopeptide models bearing the Tn or sTn, that provide control over the complexity of the chemical space of multivalent ligands. For this purpose, a concise scheme was developed for the large-scale synthesis of the Tn and sTn antigen building blocks in a relatively high yield with moderate stereo selectivity. Thiophenyl glycoside donors, in the presence TfOH/NIS or TMSOTf/NIS as promoter system, were used for the galactosylation and sialylation steps of the amino acid building block synthesis, respectively. We explored the effect of activator, temperature, solvent, and excess equivalent of sialic acid thioglycoside donor on sialylation reaction. The intended α-stereoselectivity was enhanced by adjusting the reaction conditions, which also reduced the competing 2,3-elimination process on the sialyl donor during activation, leading to overall increase in the reaction yield. These building blocks were used in the solid-phase synthesis of glycopeptide models. Using circular dichroism (CD) spectroscopy, the secondary structure of synthesized peptides was identified. The mechanistic studies by isothermal titration calorimetry (ITC) are warranted to further elucidate affinity and specificity among human lectins for synthetic Tn Antigen-Presenting MUC1 glycopeptides.

(P086) Longitudinal profiling of the Plasma Glycome from Normal and Alzheimer's Disease individuals

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Alzheimer's disease (AD) is an incurable dementia known for its neurodegenerative pathologies including amyloid beta plaques, hyperphosphorylated neurofibrillary tau tangles, and brain glucose hypometabolism. One key metabolic pathway that also utilizes glucose is glycan synthesis, which is also known to be disrupted in AD. Glycosylation is an essential protein modification which requires glucose flux through the hexosamine pathway. Prior research in our lab has demonstrated glycosylation abnormalities can be detected in the peripheral blood of early-stage AD patients. Recently, our lab has developed effective workflows to analyze glycogen and glycan content in plasma utilizing multiple mass spectrometers, including a gas chromatography mass spectrometer (GCMS) and a matrix-assisted laser desorption/ionization-mass spectrometer imaging (MALDI-MSI). In collaboration with Biobank at the University of Kentucky, led by Dr. Pete Nelson, blood plasma samples were collected at various stages of dementia status including AD (N=2), MCI (N=2), and cognitively normal controls (N=3) over a course of 11 years. These samples were subject to metabolite extraction in preparation for GCMS and MALD-MSI analysis. Utilizing these workflows, we were able to show change in abundance of several metabolite and glycan profiles overtime in both AD and MCI patients compared to our control group. Future analyses will require larger patient cohorts and full metabolic profiling to determine glycosylation differences at various cognitive stages of disease.

(P087) NMR of sparsely isotope-labeled glycoproteins provides new views of N-glycan conformation

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Structural and functional analysis of glycoproteins and their glycans has changed with recent advances in computational prediction of protein structures. Here we leverage AlphaFold predictions of the structure for an engineered version of the N-terminal domain of the cell-surface adhesion/signaling glycoprotein, hCEACAM1, to examine conformational preferences of its three N-glycans. The engineering involves introduction of a lanthanide ion binding loop that provides long-range NMR conformational data on both the protein and its glycans. Sparse isotopic labeling through supplementation of mammalian cell expression media with ^{13}C1-glucose and ^{13}C-methyl valine provides sufficient protein NMR resonances for positioning and parameterization of paramagnetic effects from the lanthanide ion, as well as NMR resonances from all anomeric and acetyl methyl carbons of glycan residues. Assignments of protein resonances and parameterization of lanthanide paramagnetic effects are made with the aid a recently released assignment tool, ASSIGN_SLP_GUI. Classification of glycan resonances to residue and linkage type using carbohydrate NMR databases allows use of paramagnetic effects to identify specific glycan interactions with the protein. These include specific interactions of acetyl methyls of GlcNAc residues with hydrophobic pockets on the protein surface.

(P088) Glycosylation-dependent regulation of F-box proteins in *Toxoplasma gondii*

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Parasites depend on a dynamic proteome to adapt to changing environments, and the Skp1/cullin-1/F-box protein/Rbx1 (SCF) family of E3 ubiquitin ligases contributes importantly to proteasome-mediated degradation. Here, we examine the role of the novel posttranslational glycosylation of Skp1 that depends on the generation of a hydroxyproline anchor by the oxygen-dependent prolyl hydroxylase PHYa, a homolog of the HIFα PHD2 oxygen-sensor of human host cells. PhyA is required for virulence by evading IFNgamma-induced nutritional immunity. Strikingly, the representation of several putative F-box proteins (FBPs) is substantially reduced in the Skp1 interactome of PHYaΔ parasites. One of these, termed FBXO13, is a predicted lysyl hydroxylase related to the human JmjD6 oncogene except for the presence of an F-box domain. The abundance of FBXO13-HA tagged at its genetic locus was reduced in PHYaΔ parasites thus explaining reduced presence in the Skp1 interactome. A similar effect in glycosylation-mutant cells, and partial rescue by proteasomal inhibitors, supported the involvement of Skp1 and the SCF. The nucleocytoplasmic localization of neither Skp1 nor FBXO13 was affected by Skp1 modification. Similar effects were observed for FBXO14-HA, a cytoplasmic protein of unknown function that is found only in apicomplexans that possess PHYa suggesting a co-evolutionary relationship. In contrast, FBXO1 was not affected by PHYa. These findings are physiologically significant because similar effects on Skp1, FBXO13 and FBXO14 were observed in parasites reared on monolayers under 0.5% O_2. The dependence of FBP abundance on Skp1 modification likely contributes to the reduced virulence of PHYaΔ parasites, which in turn may result from impaired ability to use ambient O_2 levels as a locational signal.

(P089) Close cousins of O-GlcNAc-transferases: the O-fucosyl-transferases

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Early in evolution, a gene duplication generated independent lineages of O-GlcNAc-transferase (OGT)-like enzymes, named Secret Agent (Sec) and Spindly (Spy) in *Arabidopsis* where both are now found. Members of the Sec lineage are confirmed OGTs in plants and animals. With their nearly indistinguishable sequences, Spy lineage proteins were assumed to be OGTs until the recent demonstration that Spy sequences in *Arabidopsis* and the apicomplexan parasite *Toxoplasma gondii* utilize

GDP-Fuc rather than UDP-GlcNAc as donor substrates. Here we show that Spy-like sequences in amoebozoan protists from a distant evolutionary branch, *Dictyostelium* and *Acanthamoeba*, and another apicomplexan parasite, *Cryptosporidium*, are also O-fucosyltransferases (OFTs). The O-Fuc proteins are, as for O-GlcNAc proteins, found in the cytoplasm and nucleus. Capture of O-Fuc proteins with *Aleuria aurantia* agglutinin (AAA) and proteomics analysis by nLC/MS identified numerous nucleocytoplasmic O-Fuc proteins in *Dictyostelium*, including FG-nucleoporins that parallel OFT-targets in *Cryptosporidium* and *Toxoplasma*, and OGT-targets in mammals. As for *Toxoplasma*, knockout of *Dictyostelium* OFT results in a modest growth defect under optimal growth conditions. A comprehensive search for Sec- and Spy-like sequences suggests that both genes were present in the last eukaryotic common ancestor, but that various bacteria, protists and green algae possess just one or the other, certain pathogenic fungi and animals have just OGTs, while red algae and higher plants have both. Our development of new rabbit antibodies specific for fucose-O-Ser and fucose-O-Thr (anti-FOS/T) that do not cross react with fucosylated N- and O-glycans confirm nuclear O-Fuc in *Disctyostelium*, and promise to extend and verify the gene-based predictions. The high degree of conservation of sites of deleterious mutations suggests that these forms of monoglycosylation have a shared role in regulation of responses to stress.

(P090) Docking of Glycosaminoglycans to Proteins: Methods and Challenges

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Glycoscience continues to emerge as a high-value information-rich field providing medical insight in the post-genomic era. Among the glycans, glycosaminoglycans (GAGs) represent a large family of highly sulfated, complex, linear, periodic polysaccharides that display a variety of important biological roles via interaction with protein targets. One of the recent examples is that heparan sulfate, itself a GAG, facilitates SARS-CoV-2 spike protein binding to the ACE2 receptor which triggers coronavirus infection. Not only this, but certain other kinds of GAGs have also been found to inhibit SARS-CoV-2 activity considerably and have been proposed as potential therapeutics. Computational modeling is an effective tool in studying biological systems but the nature of these long periodic linear and negatively charged polysaccharides makes it challenging to model GAG systems alone or their complexes with proteins. Docking is an essential tool for understanding protein–GAG interactions, but there has been a lack of validation studies to show the reliability of docking programs in predicting protein–GAG complexes. In this work, we will show some of the challenges and limitations of current software in modeling protein–GAG interactions by docking. [Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under award number P20GM103460.]

(P091) A human prefrontal cortex tissue microarray to study Alzheimer's disease

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Alzheimer's disease (AD) is characterized by proteinopathy, glucose hypometabolism, progressive neurodegeneration and eventually death from organ failure. One of the disease characteristics is glucose hypometabolism in the brain that can lead to perturbed cerebral glycan and glycogen metabolism. A key driver of disrupted glucose metabolism could be linked to glycans, which is a co-translational modification of proteins involved in various biological functions of the cell. Prior research has demonstrated profound glycosylation differences in AD tissue compared to normal tissue, however, these differences have yet to be linked to disease progression. In collaboration with AD biobank at the University of Kentucky led by Dr. Peter Nelson, human AD brain tissue samples from over 90 deceased patients with and without known cognitive impairment were collected, in addition to their corresponding metadata. To analyze the N-linked glycan content of the samples a Tissue Microarray (TMA) was produced using cores punched from the patients' cortex tissue, and run through an optimized, enzyme-assisted matrix-assisted laser desorption/ionization-mass spectrometer imager (MALDI-MSI) workflow. Our data not only revealed interesting correlations between glycosylation and disease pathology of AD but also interesting differences in the glycan profiles between male and female patients. Further indepth analyses will be required to further determine specific markers for the correlation between glycosylation and disease progression of AD.

(P092) Utilization of Hexosamine Biosynthetic Pathway as a Scavenger Tool to Detoxify the Glucose Toxicity under Hyperglycemia

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Diabetes is a metabolic disease often associated with hyperglycemia that results in extensive and chronic inflammation. The underlying mechanisms are not fully understood, and there is no definitive cure. Elevated intracellular glucose levels under hyperglycemia leads to toxic effects by activating multiple intermediate glucose metabolic pathways, and clinical trials using inhibitors to block these pathways in diabetic patients are not satisfactory [1]. Our studies indicate that elevated UDP-N-acetylglucosamine (UDP-GlcNAc) production by aberrant activation of the hexosamine biosynthetic pathway (HBP) is the central mechanism involved [2,3]. Under hyperglycemia, the increased intracellular UDP-GlcNAc during cell division activates two pathways: **1**) initiation of intracellular hyaluronan (HA) synthesis and ER stress/autophagy with formation of an extracellular monocyte adhesive HA matrix after division leading to inflammatory responses [4]; and **2**) activation of O-GlcNAcylation of cytosolic proteins, which serves as a nutrient sensor mediating the pathogenesis of diabetes [*5*]. Further, O-GlcNAcylation regulates both transcription and activity of HAS enzymes, suggesting that these two pathways mediate the glucotoxicity coordinately. However, heparin and its non-reducing terminal heparin trisaccharide (Hep-Tri), and 4-Methylumbelliferyl-xyloside (4MU-xyl) can prevent abnormal high glucose inside dividing cells by activating the HBP to synthesize HA and chondroitin sulfate (CS) that are secreted extracellularly [4,6]. This indicates that the HBP can be used to detoxify hyperglycemic glucose toxicity. Further, Hep-Tri lacks the deleterious side effects of heparin such as anti-coagulant activity. Therefore, **our proposed model** is**: 1**) that the elevated cytosolic glucose metabolites and UDP-sugars under hyperglycemia in dividing cells leads to ER stress and autophagy by activating intracellular HA synthesis and by increasing O-GlcNAcylation of cytosolic proteins, which induces inflammatory responses and cellular dysfunction; **2**) that heparin and Hep-Tri block activation of HA synthase in intracellular compartments during division and induce signaling pathway(s) that increase HA synthesis and formation of a monocyte-adhesive extracellular matrix after division, which prevents the cytosolic high glucose stress inside the dividing cells; and **3**) that 4MU-xyl activates CS synthesis in Golgi using UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-glucuronate (UDP-GlcUA) synthesized in the cytosol that enter the Golgi, which requires activating HBP and uronic acid pathways that prevent increased cytosolic glucose during division.

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(P093) Study of Marine Natural Products as Anti-SARS-CoV-2 Agents Using Molecular Modeling

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has created a global pandemic. Viral entry into host cells is mediated by spike glycoprotein (SGP) interactions with angiotensin-converting enzyme 2 (ACE2) and heparan sulfate glycosaminoglycans on the cell surface. Carbohydrate small molecules were found to bind to the receptor binding domain (RBD) of SGP, which also interacts with ACE2, forming a ternary complex. Moreover, glycans isolated from sea cucumber and red alga species exhibited anti-SARS-CoV-2 activities, presumably by blocking viral entry mediated through SGP–heparan sulfate interactions. Here we report a collection of computational studies conducted as part of a collaborative effort to investigate the effects of marine natural products (NPs) on the wild-type and N501Y mutant SGP RBD. Starting from an X-ray crystal structure of the RBD–ACE2 complex, a model of SGP RBD was built. To investigate the static and dynamic behavior of RBD–NP interactions, blind and site-targeted molecular docking using diverse docking programs (Glide, AutoDock Vina or ClusPro)

was carried out, followed by extensive molecular dynamics simulations with two force fields (CHARMM36 or Glycam06) and binding free energy calculations. Predicted conformations of the NPs varied considerably when modeled in water or in complex with RBD. Five NP binding sites on the RBD were studied. NP binding specificities towards SARS-CoV-2 variants were explained and important RBD residues were identified. Statistical analyses of the stability of various protein–NP complexes during molecular dynamics simulations helped to differentiate *pseudo-* vs. *real-* binding sites. Our results provide significant insights into the importance of extensive molecular dynamics calculations in order to move beyond the limitations of molecular docking. [Acknowledgment: Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under award number P20GM103460.]

(P094) Comparing the Performance of Various Binding Free-Energy Calculation Approaches in Lectin-Glycan Complexes

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Protein-glycan interactions play a key role in several biological functions like cell adhesion, recognition, differentiation, metastasis, microbial infections, and immunological recognition.[1] Lectins, the specialized proteins that recognize the glycans, are often highly specific for particular glycans. These interactions are well worth studying because of the particular biological action that can result from them, but they also are challenging to quantify. Binding of glycans to lectins is usually determined by polar interactions between the glycan hydroxyl groups and the charged amino-acid residues in the protein. Such interactions are often enhanced by presence of ion or water bridging in the binding site. Moreover, recent observations demonstrate that solvent-exposed aromatic amino acid residues frequently engage in energetically-relevant CH–π stacking interactions with the glycans.[2] The multiple kinds of non-bonded interactions that are important for lectin-glycan interactions lead to challenges in using computational methods to quantify accurately the binding free-energies of lectin-glycan interactions, especially considering that calculations on systems containing glycans need to take account of the anomeric effect and the inherent flexibility along glycosidic linkages of polysaccharides. Understanding the atomic basis of lectin-glycan interactions is of interest not only for theoretical reasons, but also for the design of glycan-based therapeutics.

We assessed the performance of several free-energy calculation approaches including Linear Interaction Energy (LIE)[3], Alchemical Free-Energy (AFE)[4], MM-PB/GBSA, QM–MM/GBSA approaches[5] and Potential of Mean Force. In this presentation, we will demonstrate how these different computational techniques can be used to explore key driving forces for glycan binding, and how these techniques can assist in lectin engineering, design of selective glycan recognition molecules (antibodies/aptamers) and glycotherapeutic agents. [Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under award number P20GM103460.]

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(P095) Methodology to Identify Physiological Ligands and Functions of Mammalian Lectin Receptors

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Mammalian genomes encode approximately 100 lectin receptors. The prototype among the mammalian lectins is the Ashwell-Morell receptor (AMR), first discovered and isolated almost 50 years ago. Past research investigating the AMR and other lectins have reported their glycan linkage binding selectivity by various methods, lately using synthetic glycan array platforms. These techniques effectively identify glycan ligands of distinct lectin receptors and can quantify the differences in glycan linkage abundance among biological samples. Those findings indicate that lectin receptors typically bind their glycan as a multi-valent ligand, sometimes including multimeric lectin complexes, and with improved binding achieved by increased glycan density. Identifying physiological glycoprotein ligands requires a different approach. As we reported, endogenous glycoprotein ligands of the AMR and the role of the AMR in blood glycoprotein half-lives and clearance were not detected for decades because the methods used included glycoprotein aggregation. We found that experimental glycoprotein aggregation, often including

denaturation, generates *de novo* lectin ligands among glycoproteins that do not bind the lectin in solution. We have developed and optimized lectin affinity chromatography for isolating physiological lectin receptor ligands among non-aggregated and non-denatured blood glycoproteins. Following lectin chromatography of blood plasma, mass spectrometry identifies candidate glycoprotein ligands. In studies of lectin deficiency in the mouse, we include wild-type results by which to measure the accumulation of glycoprotein ligands. Next, we confirm the elevated abundance and increased half-lives among glycoprotein ligands using various immunological and biochemical techniques, followed by bioinformatics analyses that assist with the prediction of physiological functions controlled by each lectin receptor. Using lectins of various sources, we have found that only a small percentage of blood glycoprotein normally exists as lectin ligands at steady state. Also, each lectin receptor studied thus far has a unique ligand repertoire of between 50-500 blood glycoproteins, with no significant overlap other than that found among the Asgr1 and Asgr2 subunits of the AMR. Each lectin also controls the abundance of a subset of the hundreds of blood glycoproteins that comprise the blood proteome. Using this combined methodology, we have previously identified blood glycoprotein ligands of the Asgr1 and Asgr2 components of the AMR and have more recently identified blood glycoprotein ligands of the alpha-M Integrin and Mrc1 lectin receptors. Meta-analysis of the biological processes and pathways linked to these lectin receptors have successfully predicted the presence of previously undetected novel and disease-inducing phenotypes in lectin receptor deficient mice. We will provide examples of our methodology, its rationale and approach, and outcomes of its application.

(P096) The Macrophage Mannose Receptor (Mrc1) and Circulating Mannosidases in the Regulation of Blood Proteostasis and Disease

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The vast majority of cell surface and secreted proteins are N-glycosylated. As secreted blood glycoproteins age in the circulation, their glycan linkages are hydrolyzed from the terminal position (non-reducing end) inward by the action of circulating exo-glycosidases, progressively exposing cryptic ligands of endocytic lectin receptors. In cases analyzed thus far, the different rates of glycan remodeling among distinct glycoproteins are inversely proportional to glycoprotein half-life and abundance. This intrinsic mechanism of blood glycoprotein remodeling and clearance is targeted by pathogens in diseases including sepsis and colitis, thereby altering the expression and function of various host blood glycoproteins and modifying disease pathogenesis and outcome. To further understand this intrinsic host mechanism in normal physiology as well as in the onset of disease, we have developed and optimized a methodology to isolate and identify physiological ligands of lectin receptors including Mrc1, also known as the macrophage mannose receptor. Our approach using lectin affinity chromatography of non-denatured blood proteins in solution followed by mass spectrometry has identified over 100 physiological candidate glycoprotein ligands of the Mrc1 lectin receptor, each bearing mannose ligands (termed mannosylated herein). From meta-analyses of the data spanning biological processes and pathways involving these glycoprotein ligands, we have successfully predicted the presence of novel previously undetected phenotypes in *Mrc1*-null mice. For example, Mrc1 deficiency increased the abundance of mannosylated Renin and Angiotensin Converting Enzyme. We have linked the accumulation of abnormally high levels of these regulatory glycoproteins to age-related elevations of blood pressure. We have further measured significant accumulation of mannosylated Myeloperoxidase associated with vascular inflammation, endothelial dysfunction, and tissue damage. Other current studies are revealing the breadth of Mrc1 function in the regulation of glycoprotein levels among multiple tissues including the brain. Past studies by this laboratory revealed that similar accumulation of mannosylated glycoproteins due to defective N-glycan synthesis resulted in chronic inflammation, autoimmunity, and degenerative disease, highlighting the need of mammalian organisms to limit the abundance of mannosylated glycoproteins, particularly those bearing terminal a3 and a6 mannose linkages. Moreover, in the blood of humans, we have discovered that 25-30% of non-hospitalized overtly healthy subjects surveyed have approximately 50% of the mannosidase activity as compared to the remaining 70-75% of the human population. Interestingly, humans with lower mannosidase activities have comparably high levels of circulating mannosylated blood proteins. We are investigating the effect of elevated mannosylated protein abundance as modulated by Mrc1 and a-mannosidase activity in disease etiology.

(P097) Development of a High-Specificity Affinity Reagent for *N*-Glycosylation Detection

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Glycans play crucial roles in nearly every aspect of biological processes, and their distinct properties make them appealing as disease biomarker targets. However, due to their highly branched and variably linked nature, glycans pose a challenge for their detection, purification, and structural analysis. Although advanced analytical techniques and instrumentation have been

developed, there is still a great need for reagents with well-defined carbohydrate specificity and high affinity that can be used to interrogate and enrich biological samples. Lectenz Bio has been engineering glycan-processing enzymes and glycan-binding proteins into high-affinity glycan-binding reagents with tunable specificities. Here, we report the development of an asparagine-linked glycan (*N*-glycan) detection reagent engineered from mouse Fbs1 (Fbx2), a component of the E3 ubiquitin ligase complex. We demonstrate that the reagent binds specifically to *N*-glycosylated peptides and proteins and not to the corresponding non-glycosylated peptides and proteins.

(P098) Influenza A viruses showed distinct replication ability in the CMP-sialic acid transporter deficient human A549 cells

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The sialic acids (Sias), particularly α2,3-linked (Sia2,3Gal) and α2,6-linked Sias (Sia2,6Gal) are known as the primary receptors of influenza A viruses (IAVs). An effective recognition between the viral surface glycoprotein hemagglutinin (HA) and the sialylated cellular receptor is required to initiate viral infection. However, recent studies suggested that non-sialylated glycans can bind to IAVs but their roles in viral infection are not fully understood. Additional studies suggested that virus receptor-binding specificities may not be static and evolve rapidly, even affecting antigenic properties. In humans, the SLC35A1 gene codifies for the transporter protein, responsible for transferring CMP-sialic acid to the medial- and trans-Golgi apparatus, and therefore plays an important role in Sia2,3Gal and Sia2,6Gal expression on the cell surface. In this study, using the human lung epithelial cell line A549 as the template, a knockout (KO) mutant ΔSLC35A1, was generated using the CRISPR/Cas9 gene-editing tool. The elimination of CMP-sialic acid transporter expression observed by Western Blot resulted in a reduction of both Sia2,3Gal and Sia2,6Gal on the cells surface, which were shown by lectin binding analyses. Mass spectrophotometry was performed to describe the N-glycans that covered ΔSLC35A1 and the A549 wild type cells, showing a lacking Sias profile on the surface of the ΔSLC35A1 cells compared to the A549 wild type cells. Growth kinetic analyses for human and avian IAVs showed a significant reduction in the level of virus replication, compared to those in A549 wild type cells. Of interest, not all viruses tested were affected in their replication in ΔSLC35A1 cells, and the variations were associated with whether the seed virus was prepared in eggs or cells. Future studies will be performed to determine the specific mutations in viruses and the alternative non-Sia receptors responsible for the replication in ΔSLC35A1 cells. In summary, this study showed that non-sialylated glycans may contribute to virus infection.

(P099) Uniquely distinct – Tissue-specific proteins carrying sialylated keratan sulfate chains are Siglec-8 ligands

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Most human Siglecs, sialic acid binding immunoglobulin-like lectins, are expressed on immune cells where they drive immune inhibition or immune excitation depending on the Siglec and the ligand it engages. Siglec-8 is an immune inhibitory Siglec expressed on human eosinophils and mast cells in the periphery and microglia in the human brain. It is among the most specific glycan binding proteins, requiring a sialylated sulfated terminal galactose, Neu5Acα2-3(6-SO4)Gal, as a minimum binding determinant. Crosslinking Siglec-8 on eosinophils results in apoptosis and on mast cells it inhibits the release of inflammatory mediators, making it an inviting target for anti-inflammatory therapy for atopic diseases. Its role on brain microglia is not yet established.

In atopic disease, Siglec-8 functions to down-regulate ongoing allergic inflammation. Data indicate that endogenous Siglec-8 ligands on immune target tissues are expressed to resolve inflammatory events. To identify endogenous human tissue-level Siglec-8 ligands, we extracted Siglec-8 binding proteins from human airways and esophagus, sites of eosinophilic inflammatory diseases,

and from human brain cerebral cortex. In every case we discovered that human tissues produce Siglec-8 ligands comprised of sialylated keratan sulfate (KS) chains carried on very large proteins - 1 million Daltons (1 MDa) and larger. This is consistent with the minimal binding determinant, in that KS has the potential to carry terminal sialylated sulfated galactose. Optimal electrophoretic resolution of the ligands in each case *required* the use of highly porous gels – composite agarose-acrylamide – with the ability to resolve proteins in the 0.25-5 MDa range. Use of standard acrylamide gels, even those with low total acrylamide, were misleading both because of the lack of resolution and limited transfer. Affinity capture and mass spectrometric analysis allowed the identification of the protein carriers in each tissue, which were remarkably unique and distinct. In human airway cartilage, Siglec-8 ligand was carried exclusively on aggrecan of three sizes – 250 kDa, 600 kDa and 1 MDa. In human airway secretions (submucosal glands and mucus secretions from patient airway lavage) it was carried on DMBT1, a 1 MDa secreted protein also known as glycoprotein 340 and SALSA. In human esophagus, it was predominantly on MUC5B, a 4 MDa mucin. In brain, it was carried exclusively on a large isoform (1 MDa) of receptor phosphotyrosine phosphatase zeta (RPTPζ, phosphacan). Notably, in chronic rhinosinusitis, airway expression of DMBT1^{S8L} was significantly increased, and in Alzheimer's disease, RPTPζ^{S8L} was significantly increased. We conclude that each distinct human tissue expresses the biosynthetic machinery to create the optimal sialylated keratan sulfate chain on unique protein carriers to support Siglec-8 binding. The reason for carrier specificity within a tissue and diversity among tissues is a matter for further evaluation.

(P100) Comprehensive glycomics and sulfoglycomics study of the N-glycosylation of papillary thyroid cancer

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Background: Aberrant N-glycosylation has been widely implicated as a hallmark in tumourigenesis associating with the progression and metastasis of several human cancers. However, the N-glycosylation of papillary thyroid cancer (PTC) had rarely been well studied for its biochemical property and correlation with the cancer metastasis.

Method: In this study, we adopted a comprehensive mass spectrometry strategy to fully address the structural features of the PTC N-glycans, in particular the sulfation on monosaccharide. N-glycans released from six pairs of PTC cancer &benign biopsies were permethylated under mild condition. Sulfates preserved permethylated N-glycans were sequenced by MALDI-TOF in both positive and negative modes. Detailed antennary arrangement of monosaccharides and sulfation were confirmed by MALDI-TOF/TOF fragmentation analysis in both polarity modes. The linkage of sialylation on the N-glycans was revealed by sialidase treatments. GC-MS linkage analysis was also employed to confirm the linkage of interested monosaccharides as well as sulfates concurrently on the partially methylated alditol acetates (PMAA) derivatised from the permethylated sulfated-N-glycans.

Results: Consistent with the results of in-group works on RNAseq and Western/lectin blotting, the N-glycan repertoire of thyroid benign is dominated by core-fucosylated bi-antennary complex structures with up to two sialyl-LacNAc antennae; tri- and tetra-antennary structures are observed with low abundance. Mono-sulfation is mainly observed on their N-glycans, and the sulfate locates on the terminal galactose of LacNAc antenna. The PTC N-glycan repertoire shows elevated GlcNAc bisection on complex glycans as well as higher abundance of tri- and tetra-antennary structures with relatively reduced sialylation, the N-glycans can mainly have mono-sulfation on the LacNAc antenna as well. The sialic acids on the N-glycans from both cancer and benign are located on the terminal galactose to form linear sialyl-LacNAc antennae with α-2, 3 linkage. The linkage of sulfate is determined by cross-ring fragmentation of the terminal galactose in MALDI-TOF/TOF negative mode and GC-MS analysis, the sulfated N-glycans from both cancer and benign have 3-O-sulfate on the terminal galactose.

Conclusion: N-glycosylation of papillary thyroid cancer is precisely changed in GlcNAc bisection, sialylation and branching compared to normal tissue. The establishment of this comprehensive N-glycan-repertoire is beneficial for further investigation in the metastasis mechanism and innovation of glycoprotein based diagnostic approaches.

(P101) Localization and identification of galactosylated proteins in murine brain using recombinant ST6GAL1 and biotinylated CMP-sialic acid

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The brain glycome is unique from other organs as it is primarily composed of high-mannose N-glycans (~60%) with relatively few complex N-glycans. Galactosylated glycans represent a small fraction of brain N-glycans (~13%) and are known to have an important role in brain function through recognition by galectins, complement factors and other glycan-binding proteins. However, the spatial distribution and protein carriers of terminally galactosylated N-glycans in the brain is not well understood

due to their relatively low abundance and a limited array of tools for their study. To overcome these issues, we synthesized a biotinylated-sialic acid derivative (CMP-SiaNAz) which could be utilized by a recombinant ST6GAL1 (rST6GAL1) to effectively label galactosylated N-glycoproteins in the brain. This approach allowed us to localize these structures within brain slices using immunofluorescence and perform enrichment techniques to identify their protein carriers through proteomics. In this study, we show that 1) the enzymatic reaction is specific to galactosylated N-glycans and the biotinylated donor substrate CMP-SiaNAz is more rapidly transferred by rST6GAL1 than its natural counterpart (CMP-Neu5Ac); 2) Galactosylated glycans are present within the synapse through colocalization with markers including PSD-95; and 3) Galactosylated N-glycoproteins are enriched for proteins associated with neuron development and synapses through gene ontology analysis. This study is the first to specifically address galactosylated N-glycoproteins in the brain, and generates a new tool to further understand the role of these structures in neurodevelopment and diseases.

(P102) Integrated omics analysis of human platelets reveals aberrant glycosylation in hematological malignancies

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Myelodysplastic syndromes (MDS) are bone marrow failure syndromes resulting from dysregulated hematopoiesis and characterized by cytopenias and hematologic cellular dysfunction. Patients with MDS are also at risk of developing acute myeloid leukemia. A common, pronounced complication is severe thrombocytopenia. The residual platelets generally exhibit abnormal mean platelet volume, a decreased ability to be activated, and increased apoptosis-induced phosphatidylserine (PS) exposure contributing to the decreased platelet counts. Collectively, these defects pose severe a disposition to excessive bleeding in MDS patients. Glycosylation changes affecting platelet production and function have been reported. For example, aberrant galactosylation occurs in megakaryocyte dysplasia affecting thrombopoiesis. Here we use lectin microarray and lectin-probe Western blot to determine platelet glycan changes associated with MDS.

In the *NUP98-HOXD13* (NHD13) transgenic mouse that recapitulates the key clinical features of MDS, including thrombocytopenia and abnormal MPV, we observed significantly different glycopatterns in platelets when compared to the wild-type (WT) mouse. NHD13 platelets showed notable increases in high-mannose glycans (HHL and GNA) and α2-3-sialyllactose (ACG), with significant decreases in LacNAc (DSA, STL and LEL), T/siayl-T antigen (ACA) and Lewis^x (LTL).

A similar trend was also observed in human MDS platelets, where platelets from 25 patients were compared to a group of 21 age-/gender- comparable healthy volunteers. Importantly, principal component analysis (PCA) revealed a dramatic segregation of the platelet glycome of MDS patients from healthy volunteers. PCA analysis also revealed a separation of MDS platelets into two distinct clusters. One cluster was mainly compromised of platelets from patients with low IPSS-R scores; the other cluster notably consisted of an equal mixture of low IPSS-R and high IPSS-R score patients.

In conclusion, lectin array analysis revealed fundamental and striking alterations in the glycome profiles of normal and MDS platelets; the essential aspects of these changes appear to be reproduced in a mouse model of MDS. Moreover, the survey of clinical platelets revealed sub-clustering of MDS platelets based on lectin binding profiles and risk of clinical disease progression based on patient IPSS-R scoring. These results highlight the importance of identifying glycans which expression matches between human patients and the mouse model enabling the study of how glycans alteration affects MDS development and progression.

(P103) Sialylation of N-glycans affects the lipid secretion governed by specialized secretory lysosomal organelles through an effect on the V-ATPase

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Complex N-glycans are known to affect protein function at the cell surface, particularly through the interaction with carbohydrate-binding proteins. In contrast, little is known about the effect of complex N-glycans in the formation and function of the secretory pathway. We previously used a human organotypic platform to interrogate the roles of distinct glycan types in

tissue formation and revealed distinct changes in skin formation associated with all tested glycoconjugates. Among the most striking phenotypes, we observed the occurrence of large intracellular vacuoles in tissues generated with *MGAT1* KO cells, suggesting an essential role of complex-N-glycans in the formation of the secretory pathway in human skin cells. Here we further examined the role of N-glycans in the formation of specialized secretory pathway in human skin cells. Using mass spectrometry, we revealed a distinct effect on the secretory pathway in *MGAT1* KO cells with altered expression of proteins involved in membrane transport, endo-lysosomal, autophagosome, and Golgi formation, such as Rab11, SNX18, SLC39A8 and SLC39A14, TOM1, VAMP7, FYCO1, AP3B1 (role in Hermansky-Pudlak disease) and LYST (role in Chediak Higashi disease). In combination with the observed phenotype, the results suggested that lack of complex N- glycans compromised the part of the secretory pathway involved in specialized lipid secretion system and lamellar body formation. This effect that was confirmed by reversion of the *MGAT1* KO vacuolar phenotype by inhibition of sphingolipid synthesis using the inhibitor myriocin. Apart from the effect on the specialized lipid secretion system, the proteomic and phosphoproteomic analyses only displayed surprisingly subtle effects.

Further analysis with super resolution microscopy revealed poor separation between early endosomal markers and lysosomal markers, suggesting problems in compartmentalization of the endo-lysosomal system. This prompted the examination of the secretory pathway microenvironment which showed increased acidification. We hypothesized that this could be the result of increased v-ATPase activity and possibly a decrease in H+ exporters activity such as NHEs or TRPML1. A potential effect on the V-ATPase was confirmed using the inhibitor bafilomycin, reverting the phenotype in 2D cultures, although this effect needs to be further explored. To investigate the importance of N-glycan sialylation, we generated a series of engineered cells lacking *ST3GAL1, ST3GAL1* and *ST3GAL2, ST3GAL4, ST3GAL6,* and *ST3GAL4+ST3GAL6*. Loss of *ST3GAL4+ST3GAL6* displayed the strongest phenotype suggesting that a2,3 sialylation of N-glycans was responsible for the accumulation of intracellular vesicles. In summary, the results suggest that loss of a2,3 sialylated complex N-glycans causes hyperacidification of the endosomal environment important for the maturation and secretion of lamellar bodies in human skin cells.

(P104) Carbohydrate specific T cell stimulation by HIV envelope glycoprotein

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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 yet been developed. Thus, there is an urgent need for the generation of a protective HIV vaccine. Past and present HIV vaccine research has been focused on developing broadly neutralizing antibodies (bNAbs) through both better understanding the virus' structure and generating a protective cellular response. Several of these bNAbs recognize the glycan shield of the HIV-1 envelope protein gp120. Recent studies have demonstrated critical roles for CD4+ helper T cells in driving antibody subclass switching, affinity maturation, and effector function of antibodies to HIV. Particularly, the generation of bNAbs requires affinity maturation and somatic mutations, involving CD4+ T cell help. Therefore, recruiting T cells to help induce high-affinity, long-lasting and protective antibody response against the gp120 glycan shield is critical. However, the importance of glycopeptides as non-conventional MHC ligands for generating T cell-mediated immunity to HIV has not been addressed. Hence, we propose that human CD4+ T cells expressing T cell receptors (TCRs) that specifically recognize glycopeptide epitopes (Tcarbs) on the Env spike play a critical role in the activation of the functional/protective antibody-producing B cells targeting Env.

We provide evidence for CD4+ T cell repertoire recognizing the glycopeptide epitope on gp120 presented by the MHCII pathway and show that the epitope is strongly immunogenic in eliciting glycan-dependent cellular and humoral immune responses in mice. We also have demonstrated that glycopeptide-specific CD4+ T cells stimulate functional, protective antibody responses against HIV. Here we show that human CD4+ T cells are stimulated by gp120 glycopeptide epitopes, suggesting the presence of human Tcarbs. Our identification of gp120 glycopeptide–induced, T cell-specific immune responses in humans offers a foundation for developing future knowledge-based vaccines that elicit strong and long-lasting protective immune responses against HIV-1 infection.

(P105) O-GlcNAcylation in KLHL proteostasis pathways links upstream signals to downstream cytoskeletal dynamics and ion homeostasis

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O-linked b-*N*-acetylglucosamine (O-GlcNAc) governs a wide range of cellular processes and is dysregulated in myriad human diseases. Despite this pathophysiological significance, the key substrates and functionally important downstream effects of

O-GlcNAcylation remain incompletely understood in most cases. Previously, we discovered that O-GlcNAc directly modifies members of the Kelch-like (KLHL) protein family of ubiquitin E3 ligase adaptors. Site-specific O-GlcNAcylation of the KLHL proteins Keap1 and gigaxonin regulates their interactions with the Cul3 scaffolding protein and with ubiquitination substrates, respectively. In recent unpublished work, to be presented here, we identified an additional O-GlcNAc-modified member of the KLHL family involved in ion homeostasis. Interestingly, we found that several ubiquitination substrates of multiple KLHL proteins are themselves O-GlcNAcylated, implying a multi-level control of such processes as cytoskeletal rearrangements, kinase cascades and ion channel function. Taken together, our results indicate that O-GlcNAcylation may be a general regulator of KLHL pathways, linking upstream signals to downstream proteostasis through the conduit of E3 ligase glycosylation.

(P106) The Glycan Structure Dictionary – a community resource for frequently used glycan structure terms

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Technical advances in the last two decades have led to an explosion in the volume of glycomics and glycoproteomics data, resulting in hundreds of published papers that describe discovery in the glycoscience domain. However, the terms used by various authors in describing glycan structural features in the literature is still non-uniform, hampering efforts to harvest information across studies. To bridge this gap, we have developed a Glycan Structure Dictionary that encompasses a comprehensive list of widely used glycan terms and their definitions. The dictionary has been developed from automated text mining which generated a glycan terms list that was then annotated with definitions, PubMed IDs, cross-references, synonyms, functions, disease annotations, as well as information from Wikipedia pages, and the Essentials of Glycobiology book. The primary identifier of each structural term is GlyTouCan accession, where available. Currently, the Glycan Structure Dictionary contains over 180 glycan structure terms. The dictionary can be accessed via GlyGen Wiki, https://wiki.glygen.org/index.php/Glycan_structure_dictionary or as a downloadable dataset via the glycan data site (https://data.glygen.org/GLY_000557). The Glycan Structure Dictionary is designed to provide a standardized reference list of glycan structural terms that will help researchers to search, extract, transfer, and report glycan information efficiently. The dictionary will also help to map glycan structures described in publications to glyco-resources such as GlyGen, GlyConnect, GlyCosmos, etc., enabling curation and text mining and improve our ability to connect glycans to function. We welcome users, researchers, glycobiologists, bioinformaticists, and other scientists to submit glycan terms and help expand the dictionary. These contributions will not only help populate the dictionary but also increase the usefulness and adoption of terms frequently used by the community. A single term can be submitted using the online form at https://data.glygen.org/gsd/, or multiple terms can be submitted through a file upload mechanism (with a sample template) provided on the Glycan Structure Dictionary Wiki Page.

(P107) Galectin-anchored indoleamine 2,3-dioxygenase suppresses local inflammation

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Anti-inflammatory treatments administered systemically are associated with numerous side effects, while locally administered drugs have short-lived efficacy. Neither approach successfully modifies the underlying causality of disease. We have developed a new way to locally modulate inflammation by fusing the enzyme indoleamine 2,3-dioxygenase 1 (IDO) to galectin-3 (Gal3). A general regulator of inflammation, IDO is immunosuppressive, catabolizing the essential amino acid tryptophan into kynurenine. We demonstrated that extracellular IDO regulates innate immune cell function, and next aimed to deliver IDO into specific tissues to provide control of inflammation. However, proteins problematically diffuse away from local injection sites. To address this, we have fused Gal3 to proteins, serving to anchor potential therapeutics to tissues via binding to extracellular glycans. Fusion protein IDO-Gal3 was retained in injected tissues and joints for up to a week or more, where it suppressed local inflammation in rodent models of endotoxin-induced inflammation, psoriasis, periodontal disease and osteoarthritis. Reduction in local inflammation, disease progression and inflammatory pain were concurrent with homeostatic preservation of tissues without global immune suppression. We hereby present a new concept of glycan-anchoring of immunomodulatory enzymes for robust control of focal inflammation in multiple disease settings.

(P108) Novel neutral galactosylated and fucosylated N-glycan epitopes from protists

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The cellular slime molds or Dictyostelia are a group of Amoebozoa which form multicellular fruiting bodies out of aggregating cells and can differentiate into spores and stalk cells. In previous studies by us and others, it has been shown that the N-glycans in the most prominent *Dictyostelium* species, the genetic model organism *Dictyostelium discoideum,* derive from the $Glc_3Man_9GlcNAc_2$-PP-Dol precursor, just as in most eukaryotes. Having analysed the N-glycome of the AX3 strain in depth, we wanted to compare the range of N-glycan epitopes from other species within the same phylogenetic group, specifically *D. purpureum*, whose genome is already sequenced, and *D. giganteum*. For a very detailed qualitative analysis and isomeric separation of the single N-glycan structures, RP-HPLC and mass spectrometry techniques were applied. Fluorescent labelling at the N-glycan reducing end improved detectability and the fragmentation pattern. In order to define the glyco-epitopes unique to a species, the collected structures were subject also to enzymatic digests and chemical treatments. In *D. purpureum* we were surprised to identify a long linear galactose-modified arm linked to a β1,4 GlcNAc of the lower α1,3-antennae, whereas *D. giganteum* on the other hand has neutral N-glycans with multiple fucose residues to terminal mannose, in addition to one attached to the core GlcNAc. In terms of their anionic N-glycans, enriched on non-porous graphitized carbon, all three species have common modifications, such as sulphation or phosphorylation. Beside (methyl)phosphorylated residues, also sulphate was identified linked to mannose, but not to fucose or GlcNAc as previously detected in a range of invertebrates. These interesting results will contribute to future studies on functional tests involving glycans and their binding partners and the comparison of different Dictyostelium species within their phylogenetic groups.

(P109) Comprehensive glycomic analysis of mucinous colorectal cancer tumor tissues by N-glycan, O-glycan, and O-glycoprotease MALDI imaging mass spectrometry

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Colorectal cancer (CRC) is a leading cause of cancer death in the United States and worldwide. CRC frequently occurs when adenomatous polyps progress to form adenocarcinoma, with mucinous adenocarcinomas being a difficult to treat, clinically aggressive subtype. Analysis of N-glycans released by peptide-N-glycosidase (PNGase) F has been used to efficiently characterize N-glycosylation in tissues by MALDI imaging mass spectrometry (IMS) in multiple cancer types. A new class of bacterial-derived O-glycoproteases have been recently identified that specifically cleave mucin glycoproteins either at the C or N-terminal adjacent to the O-linked sugar. In this study, FFPE tissue from human mucinous CRC tissues were selected for combined N- and O-glycan analysis using multi-enzymatic MALDI IMS approaches on the same slides. The first enzyme digestion was with PNGase F followed by CHCA matrix for N-glycan analysis by MALDI IMS. N-glycans and matrix were removed, and tissues were sprayed with commercial O-glycanase (New England Biolabs), the O-glycoprotease/mucinase StcE (C-terminal specific) or an N-terminal specific O-glycoprotease (New England Biolabs), singly or in different combinations. A Bruker timsTOF flex MALDI-QTOF mass spectrometer was used for imaging and data was visualized in SCiLS Lab software. Preliminary data showed an increase in the number and complexity of N-glycans within mucinous tumor tissues compared to normal colon tissue. Approximately 175 N-glycan peaks were detected in the 700-4000 m/z range with tumor regions containing increases in tetra-antennary glycans, most of which contained multiple fucose residues (n=4-9). N-glycans in mucinous tumor regions also contained increased sialylation and bisecting N-acetylglucosamine. Both O-glycan and O-glycopeptide analysis revealed peaks co-localizing to specific mucinous tumor regions. O-glycanase digestion alone resulted in detection of O-glycan species with 4-10 sugar residues consisting of hexose, N-acetylhexosamine (HexNAc) and fucose in the 500-2000 m/z range. No sialylated O-glycans were detected. Analysis of the same tissue using the mucinase StcE resulted in over 50 peptide peaks in the mass range of 1000-4000 m/z, with many peaks differing by the mass of known sugars suggesting the presence of glycopeptides. The N-terminal O-glycoprotease gave distinct peptide peaks, still specific to the mucinous tumor regions. Glycan products extracted from the same tissues are being analyzed for orthogonal glycan and peptide identifications by tandem mass spectrometry. Preliminary data indicate multiple MUC2 and MUC16 glycopeptides to be present in the mucinous tumor regions. Refinement of tandem MS glycopeptide sequencing results and linkage with MALDI IMS glycopeptide peaks are ongoing. This comprehensive approach using multiple enzyme digests that target N-glycans, O-glycans, and O-glycoproteins shows promise in characterizing mucinous features in cancer and other diseases.

(P110) Development of Improved Glycosynthase Enzymes

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Understanding IgG-specific endo-β-*N*-acetylglucosaminidases (ENGases) enables chemoenzymatic remodeling of the *N*-linked glycans on the Fc region. These glycans are important for antibody-mediated effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Accordingly, they provide an important point of regulation that can be used to improve monoclonal antibody therapeutics. One such IgG-specific ENGase, EndoS2, acts on complex, high-mannose, and hybrid type glycans by cleaving between the 1st and 2nd core *N*-acetylglucosamine (GlcNAc) saccharides. EndoS2 can be made into a glycosynthase by mutating one of the residues in the catalytic triad, D184, to a methionine. $EndoS2_{D184M}$ can transfer an oxazoline-linked glycan onto IgGs. However, this glycosynthase still retains some hydrolytic activity. Thus, we sought to improve $EndoS2_{D184M}$'s glycosynthase function.

We applied our Specificity of Enzymatic Activity and Kinetics (SEAK) method to study the transglycosylation reactions of recombinantly expressed $EndoS2_{D184M}$, using deglycosylated Rituximab and oxazoline linked sialylated bi-antennary complex type (SCT) N-glycan (ox-S2G2) as substrates. We established that introducing seleno-methionine in the active site to produce $EndoS2_{D184SeMet}$ increases the rate of transglycosylation, while decreasing the residual hydrolysis rate. In addition, we found that mutations in a non-catalytic residue in the active site, I185, also affect glycosynthase activity. These effects are likely due to subtle conformational remodeling of the enzyme's active site that results in improved transglycosylation and reduced residual hydrolysis.

(P111) Regulation of cytokinetic abscission by *O*-GlcNAcylation

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Cytokinetic abscission is the physical separation of daughter cells that concludes mitosis. Abscission initiation and progression are highly regulated processes. Premature abscission in the presence of incompletely segregated chromosomes can result in chromosome breaks that give rise to DNA damage and micronuclei which are hallmarks of cancer. To ensure that the onset of cytokinetic abscission is synchronized with the completion of upstream mitotic events, cells have evolved a cell cycle checkpoint known as the abscission checkpoint. Cells arrest abscission in the presence of mitotic errors such as trapped DNA in the intercellular bridge, misformed nuclear pores, under-replicated DNA, and tension at the intercellular bridge. Our lab is interested in identifying the molecular mechanisms underlying abscission checkpoint regulation, including how cells sense checkpoint triggers, and how the abscission machinery can be inhibited.

O-GlcNAcylation is the post-translational modification of Ser/Thr residues with O-linked N-acetylglucosamine. O-GlcNAc flux is driven by two enzymes, O-GlcNAc transferase, OGT, and O-GlcNAcase, OGA. Because OGT and OGA are reported to interact with key regulators of abscission, including Aurora B kinase, we tested whether O-GlcNAcylation is involved in abscission checkpoint signaling. Here we show that the enzymes OGA and OGT are required for maintaining an abscission arrest. Given the overlap in substrate specificity of phosphorylation and O-GlcNAcylation, we are investigating the role of O-GlcNAcylation on known phospho-regulatory sequences in proteins that function in abscission or abscission regulation. Using publicly available resources including *The O-GlcNAc Database* (www.oglcnac.mcw.edu), combined with structure function analyses, we are developing hypotheses for how O-GlcNAcylation may regulate key pathways and proteins that maintain faithful cytokinetic abscission.

(P112) Non-saccharide GAG mimetics as inhibitors of cathepsin G

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Cathepsin G (cat G) is a pro-inflammatory neutrophil serine protease that is important for host defense but also implicated as a runaway protease in inflammatory disorders including cystic fibrosis, rheumatoid arthritis, chronic obstructive pulmonary disorder, and pulmonary fibrosis. Other proteases such as neutrophil elastase (NE) and plasmin are also known to exhibit pronounced detrimental effects in these disorders. Thus, it is expected that polypharmacy using pleiotropic inhibitors would be very valuable in the management of such multi-protease dependent inflammatory disorders. Unfortunately, no inhibitors have been approved for clinical use as yet. We reasoned that the presence of basic domains on each of these proteases should be

advantageously targeted by heparin-based molecules. Yet the heterogeneity and bleeding inducing tendency of heparin enhances the risk of using its analogs as therapeutic agents in inflammatory disorders. Non-saccharide GAG mimetics (NSGMs), which are fully synthetic and homogeneous molecules that have an aromatic scaffold in lieu of the carbohydrate scaffold, have been demonstrated to allosterically modulate the activities of GAG-binding proteins with high selectivity profiles. Thus, we hypothesized that screening an in-house library of synthetic NSGMs may help identify a molecule that preferentially inhibits pro-inflammatory proteases, e.g., cat G, NE and plasmin, but not coagulation proteases such as thrombin and factor Xa.

A library of 28 NSGMs was screened against cat G using a chromogenic substrate hydrolysis assay. This led to the identification of nano-molar to micromolar inhibitors of cat G with varying levels of efficacy. Structure activity relationship (SAR) studies indicated a strong dependence of inhibitory activity on the type of hydrophobic scaffold as well as the number and positions of sulfates groups. Michaelis-Menten kinetics indicated an allosteric mechanism of inhibition, a phenomenon also observed with GAGs. Computational studies identified a putative GAG-binding site on cathepsin G using Cardin-Weintraub rules followed by GOLD-based docking and scoring studies. Comparison with our earlier results on human neutrophil elastase and plasmin identified an NSGM agent that simultaneously inhibited all three pro-inflammatory proteases with reasonable potency. Interestingly, the lead NSGM had only a marginal effect on coagulation enzymes. These results are very promising since a marked inhibition of three enzymes with low to no bleeding risk is likely to more effectively negate excess enzymes generated under pro-inflammatory conditions.

(P113) Multiplex Mass Spectrometry Imaging of N-Glycans, Glycogen, Lipid, and Small Metabolites

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Complex carbohydrates, lipids, and other small metabolites all play important roles in different cellular processes such as bioenergetics, protein structure and function, and cell signaling. These metabolites show unique regional and spatial distribution within tissue, giving insight into cellular metabolism. Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) is a technique that allows for in situ detection of analytes with spatial distribution. Through the use of different enzymes and matrices, the metabolite of interest can be imaged and spatially mapped. Our workflow details the MALDI-MSI protocol for the spatial profiling of fresh frozen tissue to image lipids and small metabolites, followed by fixation and imaging of N-glycans and glycogen from the same tissue section. As an optional step, matrix can be removed for subsequent histological staining as our method preserves tissue integrity. Overall, our multiplex workflow allows for simultaneous detection of complex carbohydrates, lipids, and polar metabolites from the same tissue.

(P114) Investigating the Effects of Human Milk Oligosaccharides in Lipoprotein Metabolism and Hyperlipidemia

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Cardiovascular diseases (CVDs) are the leading cause of death for people around the world. One type of CVD called atherosclerosis involves the accumulation of lipids and immune cells in the arterial wall that leads to the narrowing of those vessels. High levels of plasma lipids and cholesterol, known as hyperlipidemia and hypocholesteremia, respectively, are risk factors for atherosclerosis. Mouse models of hyperlipidemia, hypocholesteremia, and atherosclerosis are typically used to evaluate the effects of various compounds on the development of these maladies. Human milk is rich with bioactive compounds that demonstrate diverse biological functions including regulating development and immunity. These compounds include unconjugated oligosaccharides, which due to the diversity of their constituent monosaccharides, linkages, length, and branching leads to the production of over 150 structurally unique oligosaccharides in human milk. These individual human milk oligosaccharides (HMOs) are being examined for their potential bioactivity and resulting effects on diverse biological systems. Preliminary research indicates that a specific human milk oligosaccharide reduces atherosclerotic lesion development when administered subcutaneously in an atherosclerosis model and reduces lipid levels in plasma when administered orally in a hyperlipidemia model. Absorbed HMOs have been shown to exhibit systemic effects, but the mechanism behind this demonstrated mitigation of hyperlipidemia by HMOs is unknown. We plan to explore this mechanism by evaluating factors that influence plasma lipid levels such as lipid absorption in the gut, *de novo* VLDL production in the liver, lipid uptake in peripheral tissues, and lipoprotein clearance in the liver. We believe that these studies will elucidate potential mechanisms for

how this specific HMO is able to reduce plasma lipid levels in our hyperlipidemia mouse models and provide evidence towards the use of HMOs in treating human diseases.

(P115) Galectin-8: anti-inflammatory role during *Trypanosoma cruzi* chronic infection

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Galectin-8 (Gal-8) belongs to the group of tandem repeat galectins and is controversially involved in pro-inflammatory and anti-inflammatory processes. *Trypanosoma cruzi*, the protozoan agent of Chagas Disease (American trypanosomiasis), is responsible for the chronic cardiomyopathy as consequence of strong inflammation and fibrosis induced by the local parasite persistence. It is known that the tissue parasite load triggers the inflammatory response. *T. cruzi* presents strong myotropism to both cardiac and skeletal muscle cells. By infecting C57BL/6J (WT) and B6Gal-8 knock out (Gal-8KO) mice with *T. cruzi*, we have previously reported that in cardiac tissue obtained from infected Gal-8KO mice, Gal-8 plays an anti-inflammatory role late during the infection (doi:10.3389/fcimb.2020.00285). WT and KO mouse groups showed similar parasitemia and survival rates. Here, we show the results from the skeletal muscle (ME) study obtained 4-5 months post-infection (mpi). Tissue parasite cargo, quantified by qPCR, was similar among infected groups. However, increased inflammation was observed in ME from infected Gal-8-KO mice by histopathological studies. Through flow cytometric evaluation of ME infiltrating cells, a significant increase was observed in infected Gal8KO mice with respect to the infected WT group of: Absolute number of $B220^+$ Lymphocytes, $CD3^+$ Lymphocytes, $CD3^+CD4^+$ Lymphocytes, $CD11b^+Ly6G^+$ Neutrophils, $CD11b^+Ly6C^+$ Monocytes and $CD11c^+F4/80^-$ Dendritic cells. Furthermore, the expression of Gal-3, TGF-ß, CCL-2 and CXCL10, showed significant higher values compared to infected WT mice, evaluated by RT-PCR. The expression values of both Gal-3 and TGF-ß (fibrosis-associated molecules), are consistent with the level of fibrosis expected for this infection. Chemokines values are consistent with the increased cellularity and different populations of infiltrating immune cells observed in the infected Gal8-KO mice. These results agree with those obtained from cardiac tissue from infected Gal8-KO mice. The absence of Gal-8 allows an increase in inflammation in tissues where the parasite cargo is similar. Taking together, our studies show that Gal-8 plays a role as an anti-inflammatory molecule in the complex environment that develops during chronic *T. cruzi* infection.

(P116) An *in vivo* functional screen identifies □-2,3 sialyltransferases, ST3GAL1, and ST3GAL2 as essential for melanoma survival

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Melanoma is an aggressive type of skin cancer that accounts for most skin cancer deaths, and the incidence of melanoma has increased rapidly over the past decades. The early-stage disease can be cured by surgery; however, there is a lack of curative treatments for patients with established melanoma metastasis, resulting in significantly low survival rates. Thus, identifying key drivers in melanoma progression is essential to our understanding of melanoma biology. Glycosylation is a hallmark of cancer biology, and altered glycosylation influences multiple facets of tumor growth and progression. Herein, we conducted an innovative functional in vivo growth screen and identified the underlying α-2,3 sialyltransferases ST3GAL1 and ST3GAL2 are essential for melanoma growth. We utilized lectin microarray to identify the glycans involved in melanoma growth by comparing benign nevi and melanoma patient samples. We found glycan signatures, including an increase of α-2,3-sialosides in melanoma patient samples. Next, by examining transcriptomic datasets and melanoma tissue microarray, we found that ST3GAL1/2 and α-2,3 sialosides were upregulated in melanoma. Our study showed that ST3GAL1/2 is essential for melanoma growth in vitro. Following candidate selection, we performed proteomic analysis and identified that the CD98 heavy chain (the amino acid transporter Solute Carrier Family 3 Member 2) could be responsible for promoting melanoma proliferation. Overall, our multi-omics approach pinpointed CD98 could be a key mediator of the proliferative effect of ST3GAL1 and ST3GAL2 during melanoma transformation. In summary, our studies reveal glycans may act as molecular drivers that functionally contribute to melanoma biology, thus opening a novel path to developing glycan-based therapeutics to treat melanoma patients.

(P117) Galectin-8 C-terminal carbohydrate recognition domain is responsible for antigen uptake enhancement in dendritic cells

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Galectins comprise a family of mammalian lectins characterized by the presence of conserved carbohydrate recognition domains (CRDs) with an affinity for β-galactosides. Galectin-8 (Gal-8), from the "tandem-repeat" subgroup, possesses two CRDs, N-terminal (N-CRD) and C-terminal (C-CRD), covalently fused by a linker peptide. Each CRD displays distinct fine specificity: whereas N-CRD displays affinity for sialylated and sulfated glycans, the C-CRD prefers blood antigens and poly-N-acetyl-lactosamine. Therefore, Gal-8 can exert many biological functions with both domains acting in a concerted fashion, or separately, depending on the cellular context. Our group has demonstrated that Gal-8 enhances the elicitation of adaptive immune responses by acting on both CD4+ T cells and antigen-presenting cells. Recently, we found that Gal-8-glycan interaction at the dendritic cell (DC) surface results in antigen attachment and internalization, a crucial step during the initiation of a given immune response. Thus, we aim to characterize this interaction at the molecular level. To analyze the involvement of each isolated CRD and the requirement of the "dimeric" structure, we generated single N- and C-CRD recombinant proteins as well as chimeric proteins consisting of two covalently fused N-CRD (N-N) or C-CRD (C-C). Then, bone marrow-derived dendritic cells (BMDC) were incubated in the presence of either fluorescently labeled ovalbumin (OVA) or β-casein (which are used as antigens) together with each single domain (C-CRD or N-CRD), the equimolar mixture of both (C-CRD + N-CRD) or each chimera (C-C or N-N). OVA internalization was determined by flow cytometry. Both C-CRD (alone or in mixture with N-CRD) and the chimera C-C were able to enhance antigen internalization, whereas the presence of N-CRD or the N-N chimera did not affect the internalization rate of β-casein or OVA. Remarkably, the only addition of single C-CRD was sufficient to recapitulate the Gal-8 effect on antigen internalization, indicating that the "dimeric" structure is not required. Moreover, the C-CRD (but not the N-CRD) pre-attachment to the BMDC surface also facilitated antigen internalization, an effect that was prevented by the addition of the Gal-8 inhibitor lactose. In accordance, BMDCs pulsed with OVA in the presence of isolated C-terminal domain efficiently activated cognate $CD4^+$ T cells, as efficiently as native Gal-8. Taken together, these findings demonstrate that only the C-terminal domain participates in Gal-8-induced antigen internalization and subsequent presentation to T cells, providing new insights into the molecular characterization of the galectin-glycan interaction and its immunostimulatory effect.

(P118) Halofuginone-mediated repression of heparan sulfate leads to altered hepcidin and iron metabolism

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Hepcidin is a master iron-regulating hormone secreted by hepatocytes that determines plasma and total body iron content. We reported that liver heparan sulfate (HS) is a key regulator of hepatic hepcidin expression and systemic iron homeostasis. HS chains are attached to a distinct set of HS proteoglycans (HSPGs) and are important components of cells and the extracellular microenvironment. Interaction between growth factors and receptors is often modulated by the amount and degree of HS sulfation. Loss of liver HS prevents both BMP and inflammation-induced hepcidin expression as well as attenuated inflammation-induced anemia and aggravated iron loading. Recently, it was discovered that halofuginone reduces HS biosynthesis. Halofuginone, an anti-fibrotic agent, is an analog of febrifugine, an alkaloid found in the plant *Dichroa febrifuga*. It acts competitively with proline by binding the prolyl tRNA synthase (PRS) active site of the human glutamyl-prolyl tRNA synthetase, thereby limiting proline utilization during protein translation. Many HS biosynthesis enzymes and HS proteoglycan core proteins are proline-rich, which explains their susceptibility to halofuginone's translational suppression. In this study, we report on the effect of halofuginone to supress hepcidin expression and iron metabolism in vitro and in murine models. Mice were fed iron-rich (8.3 g/kg carbonyl iron) or iron-balanced diets (0.2 g/kg) and halofuginone or PBS was administered for 7 days intraperitoneally (0.04 mg/kg). A decrease in liver HS of halofuginone-administered mice was observed. In alignment with the hypothesis that halofuginone represses hepcidin expression, halofuginone injection significantly attenuated hepcidin upregulation in mice fed an iron-rich diet. Similar results were obtained in vitro. Our data suggest that halofuginone administration can affect hepcidin expression and treatment iron-restrictive anemias including anemia of inflammation.

(P119) Protein O-GlcNAcylation controls keratinocytes' cytokine secretion and their paracrine regulation of fibroblast activation and turnover

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Keratinocytes have a crucial role in regulating fibroblast functions by secreting pro-inflammatory and pro-fibrotic cytokines via paracrine effects. This paracrine regulation contributes to the dysregulated fibrosis seen in diseases such as keloid and scleroderma. In order to determine the role of protein O-GlcNAcylation in regulating secretion of cytokines by keratinocytes and the consequent impact on fibroblasts, primary keratinocytes and dermal fibroblasts were isolated from wildtype C57BL/6 mice and cultured separately *in vitro*. The keratinocytes were treated with a chemical inhibitor to either OGT (OSMI-1) or OGA (Thiamet-G) for 24 hours. After treatment, the inhibitor-containing media was removed and keratinocytes were cultured in fresh media for 48 hours. The keratinocyte-conditioned media (K-CM) was then transferred from the keratinocytes to the fibroblast cultures and after 48-hours, the fibroblasts were harvested for further analysis. Alternatively, the K-CM was processed for secretome analysis by Mass Spectrometry.

We found that in keratinocytes: 1), Chemical inhibition of OGT had no impact on cell viability but significantly impaired the terminal differentiation. 2), Secretome analysis revealed that in the OSMI-1-treated K-CM: a, the total number of soluble proteins in the K-CM was significantly decreased; b, the levels of some profibrotic cytokines including Thrombospondin-1 and Connective Tissue Growth Factor were significantly lower; c, the level of anti-fibrotic cytokine Heme Oxygenase-1(Hmox1) was increased. Furthermore, in recipient fibroblasts cultured in OSMI-treated K-CM: 3), Gene expression of the fibrosis markers including Acta2 and Collagen I were significantly downregulated. 4), Caspase-dependent apoptosis was induced. Chemical inhibition of OGA via Thiamet-G did not generate evident opposite effects in these cells. Further in vivo validation with the mouse model of skin fibrosis showed that: 1) the levels of protein O-GlcNAcylation are significantly elevated in mouse skin treated with bleomycin; 2) treatment with OSMI-1 significantly alleviated the murine skin fibrosis induced by Bleomycin injection.

These findings suggest that protein O-GlcNAcylation has a crucial role in regulating the secretion of pro-inflammatory and pro-fibrotic cytokines by keratinocytes; inhibition of protein O-GlcNAcylation in keratinocytes may alleviate the skin fibrosis by suppressing fibroblast activation and promoting their elimination by apoptosis.

We are combining secretome analysis with antibody-based cytokine assays to identify candidate cytokines that are regulated by protein O-GlcNAcylation in keratinocytes. In addition, transcriptomic analyses of the recipient fibroblasts by RNA-Seq are being carried out to explore alterations in global transcription caused by different K-CM. Lastly, further validation with *in vivo* mouse model of skin fibrosis using more fibrosis markers is undergoing.

(P120) Gaucher disease: How a rare disease provides a window into common neurodegenerative disorders.

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The rare lysosomal storage disorder Gaucher disease results from the inherited deficiency of glucocerebrosidase, encoded by *GBA1*. This enzyme has received increased notoriety through the discovery that mutations in *GBA1* are the most common known genetic risk factor for the development of Parkinson disease and related synucleinopathies. Glucocerebrosidase is synthesized in the endoplasmic reticulum, glycosylated in the Golgi and transported via its transmembrane receptor LIMP-2 to the lysosome, where it is activated at the acidic lysosomal pH. Once in the lysosome, it cleaves a glucose moiety from the lipids glucosylceramide and glucosylsphingosine. Gaucher disease is phenotypically diverse and has both neuronopathic and non-neuronopathic forms. Effective treatments for Gaucher disease have been developed that increase glucocerebrosidase levels and decrease lipid storage, although they do not cross the blood-brain-barrier. Mouse and induced pluripotent stem cell derived models are contributing to an advanced understanding of the function of glucocerebrosidase and the consequences of its deficiency. Both patients with Gaucher disease and heterozygous carriers are at increased risk of developing both Parkinson disease and dementia with Lewy bodies, although the precise mechanism for this association remains unknown. There appears to be an inverse relationship between levels of glucocerebrosidase and the aggregate-prone protein alpha-synuclein, and decreased glucocerebrosidase is seen in sporadic Parkinson disease. However, most patients with Gaucher disease and *GBA1* mutation carriers never develop parkinsonism, implicating the role of additional risk factors. Therapies that enhance glucocerebrosidase levels could prove efficacious in the treatment of forms of parkinsonism. Thus, this rare disease may provide both mechanistic and therapeutic insights directly applicable to Parkinson disease, a common and complex neurodegenerative disorder.

(P121) Development of a Web-based Virtual Screening Tool to Aid in Glycomimetic Discovery

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Protein-carbohydrate interactions play critical roles in physiological events, including, but not limited to: cell signaling, infection and disease onset, immune response, etc. Inhibition of carbohydrate binding to their target receptor proteins is therefore a core mechanism of action of many therapeutic agents. Computational methods play central roles in the rational design of small molecules that inhibit protein-ligand binding(glycomimetics), particularly aiding in the prediction of putative 3D structures and binding energies. Here, we present a web-based high-throughput virtual screening tool to aid in glycomimetic discovery. Users will be allowed to upload a 3D structure of a protein-carbohydrate co-complex and select positions for modification. For each such position, a few libraries of reactive compounds will be attached *in silico,* with their optimal binding pose and affinity computed by a docking scoring function. Glycomimetic compounds scored high in this initial prediction will be subjected to MD simulation. Average binding affinity of each simulation trajectory will be re-computed with a docking scoring function and the standard MM-GBSA protocol. Users will thus be provided with visual representation of glycomimetic binding under dynamic conditions, as well as theoretical ranking of binding affinities. In order to enhance prediction accuracy, we have been developing models to account for non-conventional interactions involving π systems, such as cation-π and CH-π, which have been added to our scoring function. Once mature, our tool will significantly aid the carbohydrate research community by prioritizing experimental effort towards the most promising targets.

(P122) N^6-amidation in lincosamide antibiotic biosynthesis is catalyzed by a peptide forming enzyme with a novel fold

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The lincosamides are a class of antibiotics, that includes the clinical agents clindamycin and lincomycin which are prescribed for the treatment of gram-positive bacterial infections. These antibiotics are made up of an unusual 6-amino 1-thio octose sugar core linked to a prolyl moiety by an amide linkage. This N6 amidation is essential for antibiotic activity and is a key divergence point in the development of new lincosamide therapeutics, including the recently discovered broad spectrum antibiotic Iboxamycin, a semi-synthetic derivative of lincomycin. In Nature this amide is installed by the action of a type III nonribosomal peptide synthetase (NRPS). The prolyl acid is activated as an AMP-ester and loaded onto the phosphopantetheine prosthetic group of a peptidyl carrier protein (PCP). This prolyl-PCP interacts with a peptide forming enzyme (PFE) which condenses the activated prolyl acid onto the amine of the thio octose sugar. While the enzymes involved in this transformation were known, the PFE domain had an unknown mechanism of action and its peptide sequence could not be assigned to any known protein family. We used experimental phasing to solve the X-ray structure of the PFE enzyme from celesticetin biosynthesis to 1.63 Å. The enzyme adopts a novel fold and utilizes an active site cysteine which acts in a transacylation mechanism to form the N^6 amide in lincomycin biosynthesis. These structural insights may lead to development of bio-based semi-synthetic methods to access new lincosamide antibiotics.

(P123) Detection of Increased Fucosylation Signatures in Advanced Metastatic Prostate Cancer Tissues using N-glycan MALDI Imaging Mass Spectrometry

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There is an urgent unmet clinical need to identify new treatments for men with advanced Pca. Resistance to initial hormone deprivation therapy results in castration resistance Pca (CRPC), and 80% of men with CRPC will develop bone metastases. A highly aggressive variant of CRPC, neuroendocrine PCa (NEPC), accounts for up to 30% of CRPC cases and has a 2-year median survival rate. Changes in glycosylation are associated with progression of PCa and developing new therapeutics to specific biosynthetic glycosyltransferases represent novel targets. A unique cohort of 20 autopsy tissues representing CRPC and NEPC bone metastases were analyzed for N-glycan distributions by imaging mass spectrometry approaches, as well as immunohistochemistry (IHC) assessment of a key glycosyltransferase, fucosyltransferase 8 (FUT8). Using MALDI IMS, we have identified a distinct N-glycan signature in PCa bone metastasis and NEPC associated with FUT8.

Rapid autopsy samples of CRPC and NEPC tissues were obtained from the Prostate Cancer Biorepository Network (PCBN) in collaboration with Dr. Colm Morrissey at the University of Washington. IHC analysis of FUT8 levels was done in these tissues, as well as complementary IHC analysis of 300 tumor microarray PCa samples from patients with varying grades and subsets of PCa. Each tissue was processed for N-glycan imaging mass spectrometry using established protocols on a timsTOF fleX MALDI

QTOF mass spectrometer. PNGaseF PRIME was used to release N-glycans, and Endo F3 glycosidase was used to cleave core fucosylated glycans. Distributions were visualized and quantified using SCiLS Lab software.

Using the N-glycan MALDI-IMS workflow we have identified a multi-fucosylated glycan signature in PCa bone metastasis tissue. In 10-15% of PCa tissues, it is typical to record a high fucose score, i.e., the cumulative number of multi-fucosylated glycans present. In the tissues of metastatic PCa that has spread to bone, we determined high fucose scores in 16 of 20 samples (80%), as well as many N-glycans with core fucose structures as determined by endo F3 digestion. FUT8 is the glycosyltransferase responsible for core fucose structures. In the 20 rapid autopsy PCa bone metastasis samples tested, FUT8 was detected in all samples by IHC, with an interesting overlap pattern of core fucosylated glycans. Utilizing the spatial localization of N-glycan MALDI-IMS we have detected a 15 to 25 percent overlap with areas of high FUT8 and specific core fucosylated glycans. No FUT8/core fucose overlap was observed with the more complex core fucosylated tetra-antennary glycans.

Recent murine studies conducted at Newcastle and Sheffield Universities (UK) further support FUT8's relationship with bone metastasis as well as the potential to pharmacologically target fucosylation. Together these discoveries provide an opportunity to use FUT8 and its core fucose counterpart as a therapeutic target for men with advanced PCa and ultimately increase survival rates.

(P124) 3'-Sialyllactose on Notch: Notch1 functions as a scaffold of O-linked, 3'-sialyllactosylated glycans

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

(P125) *N*-glycosylation of structured protein domains in cell-free reaction environments

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Synthetic glycobiology has the potential to revolutionize glycoprotein design and production by utilizing emerging glyco-engineering tools to tightly control the glycan pattern and location on target proteins. Importantly, these tools have already led to glycoprotein therapeutics that exhibit improved pharmacokinetics and efficacy, in particular via remodeling of their *N*-glycans. While *in vitro* glycan remodeling has been widely adopted, installation of preformed *N*-glycans onto acceptor proteins in cell-free reaction environments remains an understudied and challenging endeavor. This difficulty is partly driven by a dependence on complex, membrane-embedded oligosaccharyltransferases (OST) whose activity is constrained by the

accessibility of the modification site in an acceptor proteins. Indeed, the bacterial OST, PglB, from *Campylobacter jejuni*, which is the most commonly used OST in cell-free glycosylation systems, favors acceptor proteins with extended consensus sequences ((D/E)X_1NX_2(S/T), $X_{1,2} \neq$ proline) in flexible, solvent-exposed motifs. These requirements are in direct conflict with many native glycoproteins of therapeutic importance (e.g., immunoglobulin G (IgG); RNase A) whose minimal NXT consensus sequences in structured sequences are typically glycosylated via a co-translational mechanism. Hence, our previous observations that PglB can modify the structured acceptor sites of IgG and RNase A in glyco-competent *Escherichia coli* suggests that partial or complete unfolding is a key feature of PglB-mediated glycosylation in a cellular environment.

To investigate this issue more deeply, we utilized one-pot cell-free glycoprotein synthesis (CFGpS), which intimately couples transcription and translation with glycosylation in a single extracellular reaction environment. Specifically, CFGpS leverages *E. coli*-derived cell-free extracts enriched with glycosylation components including PglB and lipid-linked oligosaccharides (LLOs) bearing preformed *N*-glycans for tight control of glycoprotein synthesis, glycan identity, and glycan transfer to create a compelling synthetic glycobiology platform. Here, we show that the structured modification site in RNase A was efficiently *N*-glycosylated using the CFGpS system, but not when using an *in vitro* reconstituted system in which purified RNase A was provided as an already folded substrate. This result supports a cellular-like pre-folding mechanism for glycosylation in which a co-translational glycosylation mechanism may be unexpectedly active in CFGpS. Importantly, the ability of CFGpS to efficiently install *N*-glycans in structured acceptor protein domains should help to deepen our understanding of co-translational glycosylation outside of the cellular environment and could eventually provide a cell-free route for total biosynthesis of IgGs with custom glycosylation of the structured site that occurs natively at residue N297 within the Fc region.

(P126) Evaluation of Mass Cytometry for Glycosylphosphatidylinositol Anchor Protein Expression Changes in Congenital Disorders of Glycosylation

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Congenital Disorders of Glycosylation (CDG) are a heterogenous group of inherited genetic defects in glycosylation-related pathways. Existing clinical biochemical testing primarily focuses on N-linked and mucin-type O-linked glycosylation by the analysis of intact glycoproteins or glycomics; however, testing options for other glycosylation pathways are needed. Glycosylphosphatidylinositol (GPI) anchor deficiencies are a subset of CDG with limited biochemical testing options. Many patients with GPI-anchor deficiencies have reduced cell surface expression of GPI-anchored proteins (GPI-AP) assayed by flow cytometry; however, this is not a widely available clinical test and expression shifts can be subtle. In this study, we investigate the use of an emerging technology, mass cytometry, detect changes in GPI-AP expression across immune cell populations in GPI anchor deficiencies and additional CDG types.

Mass cytometry, or cytometry by time-of-flight mass spectrometry (CyTOF), is similar to traditional flow cytometry but utilizes heavy metal conjugated antibodies and a mass spectrometer detector to evaluate over 40 markers at single cell resolution. We analyzed a cohort of patients with GPI anchor deficiencies (6 patients: 4 PIGT-CDG, 1 PIGO-CDG, and 1 PIGN-CDG patients) and patients with defects in activated mannose production (5 patients: 4 PMM2-CDG and 1 MPI-CDG patients) with a customized immunoprofiling panel, MaxPar Direct (Fluidigm) with additional antibodies against GPI-APs (CD52, CD55, and CD59). Events were clustered into immune cell populations at the lineage and sublineage levels and analyzed for differential marker expression across these samples. Initial observations align with literature reports, including decreased CD16 expression on granulocytes and non-uniform reduction of other GPI-AP expression across cell lineages in GPI anchor deficiency patients. Additionally, PMM2-CDG, which results in deficiency of GDP-mannose, does not appear to reduce GPI-AP expression despite the multiple mannose linkage of the GPI structure. These preliminary results suggest that differential expression analysis by mass cytometry may aid in the diagnosis of GPI anchor deficiencies.

(P127) Precision photo-cross-linking of glycan—protein interactions through cell-surface glycan engineering

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Glycans and glycoconjugates mediate vital biological processes such as cell growth, differentiation, and immune recognition through selective interaction with glycan-binding proteins. Dysregulation of glycan-mediated signaling is implicated in disease states including cancer, inflammation, and pathogen infection. While the importance of glycan-mediated interactions is becoming increasingly recognized, detecting these native interactions is very challenging with current methods. Monovalent glycan—protein interactions are typically short-lived and have low affinities, making it difficult to capture and isolate interacting

partners. A multivalent display, ideally within the context of cell surfaces, is thus necessary to study relevant glycan/protein binding. Recognition of glycan ligands is also dependent on interactions with terminal sugar epitopes, glycan subclass-specific presentation, and conjugation to specific protein or lipid anchors. The complexity of glycan/glycoconjugate—protein interactions thus makes it challenging to identify the specific glycoconjugates involved in these critical biological processes and consequently there is a need for novel tools to discover and probe these interactions.

Introduction of photo-cross-linking groups into cellular glycans provides an attractive strategy to capture, isolate and analyze native binding partners involved in glycan—protein interaction complexes. Herein, we present a precision labeling and photo-cross-linking approach through cell-surface glyco-engineering using a photo-cross-linking CMP-Neu5Ac (CMP-sialic acid) derivative by exogenous enzymatic transfer. Our toolset harnesses the inherent specificity of various sialyltransferases to install sugar probes with high linkage and class specificity on to native glycans on live cells. These crosslinking probes are then used to interrogate sialic acid-based interactions between glycoconjugates and important receptors such as Siglecs, which are involved in modulation of the immune system and are heavily implicated in cancer, infectious disease, and neurobiology. This presentation will discuss the utility of this strategy for the discovery and analysis of glycan—protein interactions and as an exciting tool to improve our fundamental understanding how these interactions play a role in human health and disease.

(P128) Role for N-glycans and calnexin-calreticulin chaperones in SARS-CoV-2 Spike maturation and viral infectivity

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Functional and epidemiological data suggest that N-linked glycans on the SARS-CoV-2 Spike protein may contribute to viral infectivity. To investigate this, we created a panel of N-to-Q mutations at N-glycosylation sites proximal to the Spike S1-S2 (N61, N603, N657, N616) and S2' (N603, N801) proteolysis sites. Some of these mutations, particularly N61Q and N801Q, reduced Spike incorporation into Spike-pseudotyped lentivirus and authentic SARS-CoV-2 virus-like-particles (VLPs). These mutations also reduced pseudovirus and VLP entry into ACE2-expressing cells by 80-90%. In contrast, glycan mutations had a relatively minor effect on cell surface expression of Spike, ACE2-binding, and syncytia formation. A similar dichotomy in function was observed when virus was produced in host cells lacking ER chaperones, calnexin and calreticulin. Here, while both chaperones regulated pseudovirus function, only VLPs produced in calnexin-KOs were less infectious. Overall, Spike N-glycans are likely critical for SARS-CoV-2 function and could serve as drug targets for COVID-19.

(P129) The Role of Glycosylation in Modulating ACE2 – SARS-CoV-2 Spike Affinity

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The interaction between the SARS-CoV-2 Spike glycoprotein (Spike) and the glycosylated human angiotensin-converting enzyme 2 (ACE2) receptor is essential for viral adhesion and establishing infection. A 3D structural analysis of the Spike-ACE2 complex, combining glycoproteomic data and molecular dynamic simulations, revealed ACE2 glycosites N90, N322, and N546 interact with Spike and may influence overall binding affinity. Understanding the role of Spike and ACE2 glycosylation during infection may enhance vaccine and therapeutic development as well as shed light on how variations in susceptibility and prognosis of COVID-19 relate to glycosylation. Here, we report the impact of Spike and ACE2 glycosylation binding affinity for the circulating variants of SARS-CoV-2 by point mutations, which we quantify using biolayer interferometry.

(P130) Molecular characterization of Human-Specific *SIGLEC16P*

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CD33-related Siglecs (CD33rSiglecs) belong to a major subfamily of sialic acid binding immunoglobulin-like lectins (Siglecs) that are prominently found on immune cells. Siglec-11 and Siglec-16 are two CD33rSiglecs that have not been studied extensively,

especially in the brain. They are paired polysialic acid binding receptors with highly homologous ligand binding domains but opposing inflammatory signaling when engaged: Siglec-11 functions as an inhibitory receptor, whereas Siglec-16 exhibits activating properties. Human *SIGLEC11* underwent multiple gene conversions by an adjacent *SIGLEC16P* allele, and the converted *SIGLEC11* allele became fixed and expressed in microglia only in humans. Siglec-11 and Siglec-16 thus have uniquely human features, including their central nervous system expression, microglial Siglec-11 can be secreted in exosomes, and the pseudogenization of *SIGLEC16* (*SIGLEC16P*). Compared to the full-length *SIGLEC16* allele (*SIGLEC16FL*), *SIGLEC16P* allele is characterized by a four base pair deletion in exon 2 that leads to an early termination and is the dominant allele in the human population with an allele frequency of ~0.73. *SIGLEC16P* function has not been previously investigated. Here, we show that: 1) *SIGLEC16FL* genotype is associated with a lower risk of being admitted to the neonatal intensive care unit (NICU) for brain-related injuries, 2) transfection of the *SIGLEC16P* gene leads to the synthesis of a truncated Siglec-16 (Siglec-16P) product that is secreted in human cells, 3) Siglec-16P protein is post-translationally glycosylated, and 4) Siglec-16P binds polysialic acid in a concentration-dependent fashion. These results suggest that *SIGLEC16P* is not a null gene.

(P131) Expanding the Chemical Toolbox for Selective Cell-Surface Glyco-Engineering

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Glycans present in the extracellular glycocalyx surrounding cells are crucial mediators of a wide range of biological processes. The interactions of these complex glycans with proteins is central to a diverse range of physiological and pathological processes including cell-cell communication, molecular recognition, immunological responses, infectious diseases and cancers. However, identifying the precise endogenous glycan structures involved in these interactions and the mechanisms by which they elicit function is a major challenge. The heterogeneity and complexity of cell-surface glycans, coupled with their biosynthesis not being template-driven, make it difficult to elucidate biological function by traditional approaches. As a result, novel chemical biology tools are needed to meet the demand for new information and advance our understanding of the function of these important biomolecules.

To address these challenges, we have been working towards expanding the cell-surface glyco-engineering toolbox. We use chemo-enzymatic synthetic strategies to prepare carbohydrate-based probes and biologically relevant complex glycan structures. These probes or glycans are then installed on cells using an exogenous enzymatic cell-surface glyco-engineering methodology to investigate how these structures interact with glycan-binding proteins and modulate cell function. This glyco-engineering strategy has allowed for structure-function analysis of glycans on cells to answer fundamental questions on the biology of these molecules. We have also demonstrated how this approach can be used for precision installation of carbohydrate probes to interrogate and capture glycan-protein binding partners. Here, we will discuss chemo-enzymatic approaches to synthesize chemical probes and complex glycans. We will also describe studies using glyco-engineering to probe and interrogate the recognition of glycans and glycoproteins on cell surfaces. These approaches will enable the identification of functional glycan ligands of glycan-binding proteins as targets for therapeutics and will facilitate the development of glycan-based strategies for combatting disease.

(P132) GlycoEnzOnto: A GlycoEnzyme Pathway and Molecular Function Ontology

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The 'glycoEnzymes' include a set of proteins having related enzymatic, metabolic, transport, structural and cofactor functions. Current there is no established ontology to describe glycoEnzyme properties and to relate them to glycan biosynthesis pathways. We present GlycoEnzOnto, an ontology describing 386 human glycoEnzymes curated along 135 glycosylation pathways, 134 molecular functions and 22 cellular compartments. The pathways described regulate nucleotide-sugar metabolism, glycosyl-substrate/donor transport, glycan biosynthesis, and degradation. The role of each enzyme in the glycosylation initiation, elongation/branching, and capping/termination phases is described. IUPAC linear strings present systematic human/machine readable descriptions of individual reaction steps and enable automated knowledge-based curation of biochemical networks. All GlycoEnzOnto knowledge is integrated with the Gene Ontology (GO) biological processes. Using this ontology, we performed a systems-level, glycoinformatics analysis of The Cancer Genome Atlas (TCGA) to investigate the relation between glycoEnzyme transcript levels, cancer type and glycosylation pathways. This dataset includes over 11,000 patients and 33 different human tumor types. The analysis revealed the power of the new ontology to segregate various cancer types based on tumor origin, molecular subtype and dysregulated pathways. It demonstrated that GlycoEnzOnto enables improved

transcript overrepresentation analyses and glycosylation pathway identification compared to other available schema, e.g. KEGG and Reactome. Overall, GlycoEnzOnto represents a holistic glycoinformatics resource for systems-level analyses. **Resource availability**: https://github.com/neel-lab/GlycoEnzOnto

(P133) Apolipoprotein glycoforms and the postprandial lipid milieu

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Background: Postprandial lipemia is closely associated with endothelial dysfunction and cardiovascular disease. Apolipoprotein glycosylation likely influences the postprandial lipemic milieu.

Objective: To evaluate the relationships between postprandial lipid metabolism and various glycoforms of apolipoproteins B, CIII, D, H, and M.

Methods: In 24 women (age: 50.4 ± 11.4y, BMI: 32.2 +/- 4.2 kg/m2) with dyslipidemia, insulin resistance, or both, fasting and postprandial (30, 180, and 360 min) lipidomic responses to a high-fat meal challenge test (60% fat, 25% carbohydrate, and 15% protein) were measured using a CSH-QTOF MS/MS protocol. Fasting glycoforms of apolipoproteins (apo) B, CIII, D, H, and M were measured using a UPLC-MS/MS protocol. Missing data (5%) were imputed and transformed to achieve normality. The fasting and postprandial lipidomic data (492 variables) were used in an iterative PCA-based multi-collinearity reduction algorithm to identify 68 variables that exhibited significant orthobliqueness. Using a one-compartment single oral bolus non-linear curve fit algorithm, we estimated the "appearance", "area under the curve (AUC)," and disappearance" rates of these lipids in plasma. Apolipoprotein glycoforms at fasting were used to predict the appearance and disappearance rates using a stepwise regression model (minimum BIC, forward direction) with FDR correction, variable inflation assessment, and standardized beta-weight estimation.

Results: ApoD-Hex(7)HexNAc(6) and ApoD-Hex(9)HexNAc(8) were inversely associated with the appearance rate of ceramide d42:2 and glucosylceramide d42:2, respectively, while being positively associated with the disappearance rate of the same. ApoD-Hex(6)HexNAc(5)Fuc(3), however, was positively associated with the appearance rate of ceramide d42:1 and inversely associated with the disappearance rate of ceramide d43:1. ApoCIII- Hex(1)HexNAc(1)Fuc(1)NeuAc(1) was inversely associated with the appearance of Acylcarnitines (C14:2 and C18:1) and AUC TAG(46:2). Apo CIII-Hex(1)HexNAc(1)NeuAc(2), on the other hand, was positively associated with the disappearance rate of TAG(46:2) and inversely associated with AUC TAG(46:2). ApoCIII Hex(1)1HexNAc(1)NeuAC(1) was positively associated with the appearance rate of acylcarnitine (C18:1) but inversely associated with the disappearance rate of TAG(46:2), DAG(36:2) and TAG(56:4).

Conclusions: Fucosylated ApoD glycoforms, but not non-fucosylated glycoforms, are associated with a ceramide-rich plasma which is in turn linked to a higher risk of endothelial dysfunction. The disialylated glycoform of Apo CIII, as has been shown earlier, is associated with the disappearance rate of triglyceride species from plasma and likely also influences lipid oxidation, both of which are associated with modulating cardiovascular disease risk.

(P134) Novel CLC-Kb pore mutation associated with defective glycosylation and renal tubulopathy

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Bartter's and Gitelman's syndromes are inherited autosomal recessive conditions resulting in defects of renal electrolyte handling with salt wasting and accompanying hypokalemic metabolic alkalosis. Mutations in the CLCNKB gene (1p36), encoding a basolateral chloride channel ClC-Kb, have been identified as the most frequent cause of classic (type 3) Bartter's syndrome. Our laboratory identified a family with a mixed Bartter's/Gitelman's phenotype and end-stage kidney disease in the absence of nephrocalcinosis. Whole exome sequencing identified, and sanger sequencing confirmed a novel homozygous mutation in the CLCNKB gene (nt G499T, aa G167C) of the proband and his affected brother and two heterozygous, unaffected parents. Mechanistic studies revealed that this highly conserved amino acid substitution (Gly-167-Cys) in the pore of the channel inhibits its function with preserved surface expression of the mutant protein in native kidney tissue. This mutation unexpectedly

causes a defect in glycosylation of the encoded gene product CLC-Kb, although the mutated residue is located distant from the glycosylation sites. Additional studies reveal a role for glycosylation to promote channel function, independent of changes in surface expression. This case reveals a novel regulatory mechanism for CLC-Kb function that may help to understand the role of CLC-Kb channel defects in blood pressure and kidney disease.

(P135) Glycan Microarray Services and Bioinformatics through the National Center for Functional Glycomics

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The National Center for Functional Glycomics (NCFG) at BIDMC at Harvard Medical School (HMS) is an R24 National and Regional Resource Center, funded by the National Institutes of Health, NIGMS. The NCFG is an outgrowth of the successful Consortium for Functional Glycomics (CFG) glycan microarray resources. The overall goals of the NCFG focus on providing needed resources and technologies for defining protein-glycan interactions to the community. Our central discovery platform is glycan and glycopeptide microarrays, with glycans derived from chemo-enzymatic synthesis (*defined microarrays*) and natural animal sources (*shotgun microarrays*), and microbially-derived components. A set of glycan components will be translated to the Luminex platform to allow for additional binding capabilities. Additional linker strategies are in development to allow such features as reversibility, bifunctionality, and natural amino acid linkages. We maintain legacy array data in addition to newly generated data, and are working with GlyGen to synchronize datasets and allow for integration of new datasets into the repository. We also develop and utilize bioinformatics capabilities such as: GLycan Array Dashboard (GLAD) which provides tools for analyzing and comparing glycan array data and visualization capabilities; GlycoGlyph which enables drawing of glycans with SNFG formatting with ease, export capabilities, and links to other databases and features; and Glybrary, a newly developed comprehensive database for inputting and linking sample and assay details, connecting datasets, and tracking projects, with the goal of replacing the stagnant *CFG* website. These programs are or will be publicly accessible through the NCFG website-http://ncfg.hms.harvard.edu/home

The paramount service offered through the NCFG is the analysis of glycan binding proteins (GBPs) on our collection of glycan microarrays, as fee-for-service and through collaborative research. The number, diversity, and biological relevance of both defined and shotgun glycan microarrays is continuously growing. New requests for defined or shotgun microarrays and other printing projects can be directed to the NCFG. Our tangential work through the BIDMC Glycomics Core and Cummings Lab includes Glycomic analyses using advances mass spectrometry methods, and development of robust anti-glycan reagents using the lamprey system. Overall, we focus on technologies aimed at defining cellular glycomes important in human biology and disease, and we aim to support the community in their endeavors into defining protein-glycan interactions. *R24GM137763*

(P136) TFCP2 is a novel transcriptional regulator of heparan sulfate assembly and growth factor binding in human melanoma

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Heparan sulfate proteoglycans (HSPGs) are ubiquitously expressed on all animal cells and in the extracellular matrix. Each HSPG consists of a core protein with one or more covalently attached linear heparan sulfate (HS) chains composed of alternating glucosamine and uronic acids that are heterogeneously *N*- and *O*-sulfated. These complex carbohydrates regulate many important biological processes including cell proliferation and development through their interaction with a large number of matrix proteins and growth factors. The arrangement and orientation of the sulfated sugar residues of HS specify the location of distinct ligand binding sites on the cell surface, and these modifications can vary temporally during development, spatially across tissues, and in disease states, such as cancer. While most of the enzymes involved in HS biosynthesis have been studied extensively, much less information exists regarding the regulatory mechanisms that give rise to the variable composition and binding properties of HS. To search for novel regulators of HS biosynthesis, we performed genome-wide CRISPR/Cas9 screens in human cancer cells and uncovered the alpha globin transcription factor, TFCP2, as one of the top hits. In this study, we knocked out expression of TFCP2 in human melanoma cells to investigate its role in the regulation of HS assembly. *TFCP2* inactivation suppressed cell growth and modulated the expression of multiple enzymes involved in HS assembly, including the extracellular endosulfatase SULF1, which differentially affected the interaction of heparan sulfate-binding proteins. Similarly, pharmacological inhibition of TFCP2 activity altered HS assembly and growth factor binding. Overall, these studies provide insight into the molecular mechanisms by which HS biosynthesis is differentially regulated in normal physiology and disease and identify a new potential target for melanoma.

(P137) Structural basis of glycan recognition by eukaryotic oligosaccharyltransferase

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Oligosaccharyltransferase (OST) is an essential membrane protein complex in eukaryotes that catalyzes the transfer of pre-assembled $GlcNAc_2$-Man_9-Glc_3 from a dolichylpyrophosphate (Dol-PP) carrier to asparagine residues of secreted proteins in the lumen of the endoplasmic reticulum. Attachment of the fully assembled glycan to newly synthesized proteins is required for downstream processes that define the fate of the protein to folding or degradation. Despite the recent advances in the structural investigation of OST complexes, the molecular basis of glycan recognition has remained elusive. Here we reconstituted the complete biosynthetic pathway of eukaryotic lipid-linked oligosaccharide (LLO) starting from chemically synthesized precursors and purified glycosyltransferases (ALG enzymes). The chemo-enzymatically produced LLO analog, Dol20-PP-$GlcNAc_2$-Man_9-Glc_3, was used for biochemical and structural studies of LLO glycan recognition by yeast OST. We found that two regions of the LLO glycan are recognized by OST at distinct binding sites. Whereas the $GlcNAc_2$ moiety is recognized close to the active site of the catalytic subunit STT3, the terminal glucoses (Glc3) are accommodated in a binding pocket formed by non-catalytic subunits. Our results describe the basis of LLO-glycan recognition and assign functional roles to auxiliary OST subunits.

(P138) M021: Pompe Enzyme Replacement Therapy with Highly Phosphorylated Oligosaccharides by the Co-Expression of Acid Alpha-Glucosidase with S1S3 GlcNAc-1-Phosphotransferase.

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Pompe disease is a rare inherited metabolic disorder of defective lysosomal glycogen catabolism due to a deficiency in acid alpha-glucosidase (GAA). Alglucosidase alfa enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA ERT) has provided clinical benefits, but these benefits have been limited primarily due to poor drug targeting of ERT to skeletal muscles. Recent therapeutic approaches (Cipaglucosidase alfa and Avalglucosidase alpha) have improved cation-independent mannose-6 phosphate receptor (CI-MPR) targeting by increasing the number of phosphorylated oligosaccharides present on rhGAA. Cipaglucosidase alfa is produced using a unique CHO cell line that produces rhGAA with increased phosphorylated oligosaccharides coupled with a highly engineered manufacturing process that yields an ERT with at least one bis-phosphorylated oligosaccharide per molecule of protein. Avalglucosidase alpha is a chemically engineered rhGAA produced by attaching synthetic bis-phosphorylated oligosaccharides to rhGAA utilizing a chemical conjugation process exploiting oxidized sialic acid residues.

M021 is a novel rhGAA co-expressed with S1S3-phosphotransferase in CHO cells being developed by M6P Therapeutics. By utilizing the S1S3-phosphotransferase, GAA phosphorylation is greatly enhanced in the production cell line. Since phosphorylation is no longer a challenge, this more allows more focus on optimizing the entire oligosaccharide profile of M021. Currently we have developed a cell line and process that yields a rhGAA that has >90% of its oligosaccharides phosphorylated with >70% being bis-phosphorylated. This is of importance since bis-phosphorylated have the greatest affinity for the CI-MPR. In addition to the high level of bis-phosphorylated oligosaccharides, M021 have very low levels of neutral oligosaccharides (<5%). This unique oligosaccharide profile should maximize CI-MPR targeting while minimizing unproductive mannose receptor and a**sialoglycoprotein** receptor clearance in the liver.

(P139) N-glycoproteomic profiling of phosphoglucomutase 2 (Pgm2) knockout mouse heart reveals distinct glycosylation pattern

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Introduction: Phosphoglucomutase-1 (PGM1) catalyzes the interconversion of glucose-1 phosphate and glucose-6 phosphate and plays a fundamental role in carbohydrate metabolism. Due to its essential role in monosaccharide activation, autosomal recessive PGM1 deficiency leads to a Congenital Disorder of Glycosylation (CDG). PGM1-CDG is the third most common

N-linked CDG, presenting with early onset, resistance to therapy, and frequently lethal dilated cardiomyopathy. The exact consequences of PGM1 deficiency on glycosylation of proteins expressed in the heart have not yet been investigated.

Methods: To assess the alterations in glycosylation in PGM1-CDG, we compared the proteome and glycoproteome of heart tissue from wild type mice and those with cardiomyocyte-specific conditional knockout (cKO) of *Pgm2* (mouse ortholog of human *PGM1*) gene. The extracted proteins were digested into peptides and subsequently labeled with tandem mass tags (TMT) for a multiplexed analysis. These labeled peptides were fractionated using basic reversed phase liquid chromatography for proteomics and the glycopeptides were enriched and fractionated using size exclusion chromatography. The samples were analyzed by LC-MS/MS on a high resolution Fourier transform Orbitrap Eclipse mass spectrometer. Data analysis was done using Proteome Discoverer and pGlyco software.

Results: Quantitative proteomics analysis of left ventricular cardiac tissue obtained from *Pgm2* cKO mice revealed no dramatic alterations at the level of global protein expression although the expression of Pgm2 protein was reduced by ~80%. Some of the proteins that exhibited significant changes in abundance (p-value <0.05) are known to be involved in lipid and energy metabolism including corticosteroid-binding globulin, mitochondrial NAD(P) transhydrogenase and mitochondrial D-2-hydroxyglutarate dehydrogenase. The glycoproteomic analysis showed an overall decrease in protein N-glycosylation. Of the 1,640 individual N-glycopeptides identified, 171 glycopeptides were significantly reduced in cKO samples. Significantly, we observed widespread changes in glycosylation levels of 74 glycopeptides derived from in multiple subunits of laminin including alpha-2, alpha-4, alpha-5, beta-1, beta-2 and gamma-1 subunits.

Discussion: Because a number of proteins associated with cardiac contractility are glycosylated, our findings suggest that decreased glycosylation could impact cardiac function in PGM1-CDG. We hypothesize that aberrant glycosylation of multiple laminin subunits adversely affects its ability to interact with integrin and/or α-dystroglycan, thereby impairing the integrity of cardiomyocytes, which could contribute to development of dilated cardiomyopathy in *Pgm2* cKO mice.

(P140) Postnatal development of carbohydrate-specific antibodies

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Carbohydrate-specific antibodies are common among all classes of immunoglobulins. Various carbohydrate-specific antibodies, including antibodies to ABO blood group antigens, develop upon contact of immune cells with gut microbes, which express a vast diversity of carbohydrate antigens. In fact, the development of carbohydrate-specific antibodies coincides with the microbial colonization of the gut at birth. Before birth, IgG are transferred from the maternal circulation to the fetus through the placenta. Postnatally, additional maternal IgG and IgA are transferred to infants through breastfeeding. A large pool of serum IgM and IgG recognizes bacterial carbohydrate antigens, including α-rhamnose, α-Gal and GalNAc(α1-3)GalNAc epitopes, but also glycan motifs common on host cells. Given the importance of the gut microbiota in inducing carbohydrate-specific antibodies, we analyzed global antibody specificities towards host glycans and bacterial glycoconjugates using glycan arrays. As expected, we found that carbohydrate-specific IgM are absent in cord blood, whereas limited cord blood IgG reactivity to carbohydrates was detectable. The reactivity of IgG to specific gut bacterial antigens was similar in matching cord and maternal blood samples, thus pointing to an efficient maternal transfer of anti-microbial IgG. The production of carbohydrate-specific IgM and IgG in infants rapidly increases in the first weeks of life and reaches nearly adult-levels already by six months of age. In addition to early vaccination, such as by administration of *Haemophilus influenzae* type b conjugate vaccine, the development of carbohydrate-specific antibodies induced by gut microbes enhances the natural protection of infants towards a broad range of glycosylated pathogens.

(P141) Applying an Optimized Liposome Formulation to Dissect Siglec–Glycolipid Interactions

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Siglecs (sialic acid-binding immunoglobulin-type lectins) are a family of cell surfaces receptors immune cells use to tell friend from foe. Cells communicate to Siglecs that they are friendly via sialylated glycoproteins and glycolipids. Gangliosides are a

family of sialylated glycolipids in mammals and are found ubiquitously across all cell types and tissues. Traditionally, Siglec–ganglioside interactions have largely been studied outside the context of a bilayer while ideally, they should be studied in their natural environment of a lipid bilayer. Here, we optimize a liposomal nanoparticle formulation for dissecting Siglec–ganglioside interactions and then use this formulation to screen the entire human Siglec family against a panel of gangliosides. Many known Siglec–ganglioside interactions were reproduced, and we identified serval novel Siglec-ganglioside interactions. However, the most novel interactions were with Siglec-6. Moreover Siglec-6 engages glycolipids independent of its conserved arginine residue, a trait unique to Siglec-6. The specificity of this novel binding site was then probed with a panel of synthetic neoglycolipids which led to the development of a neoglycolipid with higher avidity to Siglec-6 compared to natural gangliosides. Using liposomes formulated with this neoglycolipid we were able to target Siglec-6 on physiologically relevant cells and tissues such as memory B-cells and placental syncytiotrophoblasts. We also demonstrated that Siglec-6 facilitates the binding and internalization of extracellular vesicles, placing Siglec-6 in a unique opportunity to serve a broad immunological role.

(P142) Improved recovery of glycans by salt in EZGlyco® O-Glycan Prep Kit and its application to membrane-transferred glycoproteins

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O-glycans are of interest in the research for biomarkers focusing on glycan alterations caused by a specific disease, therefore, a simple and accurate sample preparation method has been required. EZGlyco® O-Glycan Prep Kit enables safe, simple and rapid sample preparation including the liberation of O-glycans from glycoproteins, purification, and fluorescent labeling. The O-glycan release is based on a chemistry named "eliminative oximation", which significantly reduces the decomposition of reducing oligosaccharides when liberated in the absence of a reductant, also known as peeling reaction, than traditional hydrazinolysis method.

In this study, we found that a small amount of salts such as NaCl in the reaction solution significantly improves the O-glycan recovery, and established a workflow of O-glycan sample preparation from a trace amount of glycoproteins transferred onto a membrane. First, the effect of salt on O-glycan recovery with the Kit was assessed in the liquid-phase reaction. Next, we examined the effect of salt on the recovery of O-glycans from a PVDF membrane which fetal bovine fetuin was immobilized using the Kit. LC-MS analysis demonstrated that the novel combination can be applied for O-glycan preparation workflow, *i.e.* liberation, purification and fluorescent labeling, from glycoproteins those immobilized on a membrane as well as in liquid-phase reactions. The presented technique greatly improves the robustness of O-glycan analysis from trace amounts of glycoproteins, thus expected to be a useful tool in exploring a new glycan biomarker when employed with proteomics methods in unison.

(P143) Upregulated expression of the ST6GAL1 sialyltransferase in tumor cells creates sialoglycan ligands for immunosuppressive Siglec receptors

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Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest human cancers. Immune evasion is one of the critical factors for therapeutic failure and poor prognosis. During tumorigenesis, glycans on cancer cells bind to lectin receptors on immune cells, causing immune cell inactivation. In particular, sialic acid–binding immunoglobulin-type lectins (Siglecs) play a crucial role as immune checkpoints in various cancers including PDAC. However, while much research has focused on the effects of Siglecs on immune cell behavior, limited attention has been paid to tumor-associated changes in sialoglycans that serve as ligands for Siglecs. One key glycan change noted in cancer is an increase in the α2-6 sialylation of *N*-glycosylated surface receptors, a modification added by the ST6GAL1 sialyltransferase. ST6GAL1 is upregulated in many cancers including PDAC. Our lab generated a mouse model of PDAC with either pancreas-specific knock-in of oncogenic K-ras (KC mouse) or K-ras in combination with ST6GAL1 knock-in (KSC mouse). Compared to KC mice, KSC mice display accelerated PDAC progression. In the current study, we performed flow cytometry experiments with Siglec-Fc fusion proteins on pancreatic cancer cells with differential ST6GAL1 expression in order to determine whether ST6GAL1 activity creates ligands for Siglecs. We found that the overexpression of ST6GAL1 in 266-6 pancreatic cancer cells induced an increase in sialoglycans recognized by Siglecs 2,3,9 and 10, whereas ST6GAL1 knockdown decreased binding by these same Siglecs. In contrast, ST6GAL1 activity had no effect on the binding of Siglec 1, which is specific for α2-3 sialic acids. Similar results were observed for Suit2 pancreatic cancer cells, and for the HPNE nonmalignant pancreatic acinar cell line. Next, we evaluated Siglec reactivity using a mouse model wherein ST6GAL1 alone was knocked into the pancreas (SC mouse). Analyses of Siglec-Fc binding to acinar cells isolated from WT or SC pancreata corroborated results from established cell lines. Based on these data, we hypothesize that increased α2-6 sialylation on tumor cells promotes dampening of immune response and accelerated PDAC progression via Siglec signaling. Interestingly, with the exception

of Siglec 2, Siglecs 3, 9 and 10 are mostly expressed on myeloid cells and macrophages. Moreover, macrophages polarized to an immunosuppressive M2 phenotype are key contributors to PDAC progression. Thus, we conducted immunohistochemistry (IHC) on pancreata from KC and KSC mice, and found an increase in CD163 positive immunosuppressive M2 macrophages in KSC mice. Additionally, IHC using Siglec-Fc proteins revealed that KSC pancreata have higher levels of sialoglycan ligands for Siglec 3, as well as the murine counterparts for Siglecs 9 and 10, Siglecs E and G, respectively. Collectively, these results reveal a potential mechanism for suppressing macrophage cell response in an ST6GAL1-mediated hypersialylated PDAC tumor microenvironment.

(P144) Novel Heritable Human COSMC Mutation Results in a Multisystemic Chaperonopathy

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Cosmc is a specific endoplasmic reticulum-localized molecular chaperone responsible for folding T-synthase, which is the enzyme required for extended O-glycans in animal cells. Defects in this pathway result in the expression of truncated O-glycans, whose expression is invariably associated with many disease pathologies in humans and mice. Recently, we identified a novel heritable genetic mutation in X-linked *COSMC (C1GALT1C1)* in two male patients. Both patients carry a mutation (c.59C>A - p.Ala20Asp, A20D) within the predicted transmembrane domain (TMD) of Cosmc, a type II transmembrane protein. The affected patients have short stature, developmental delay, thrombocytopenia, intellectual disability, and acute kidney failure of unknown origin. Collectively, for this diverse spectrum of disease phenotypes with a Congenital Disorder of Glycosylation (CDG), we have termed them *Cosmc*-CDG. Our deeper biochemical characterization of the immortalized lymphoblastoid cells from a patient showed remarkable loss of Cosmc protein and correspondingly significant loss of T-synthase enzyme and its activity (<5%), which could be partly restored by transient transfection of wild type Cosmc. Our analysis of extended O-glycans from these patients' immortalized cells showed a majority (~90%) of normal O-glycans and some truncated structures (Tn antigen), perhaps explaining the patient's developmental viability.

(P145) Macromolecular Circulating Immune Complexes in IgA Nephropathy

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IgA nephropathy (IgAN) is the most common kidney disease worldwide, leading to terminal renal failure in ~30-40% of affected individuals within 10-20 years after diagnosis. IgAN is defined by the detection of IgA1 in kidney biopsies that occurs as a result of the deposition of circulating immune complexes containing autoantibodies against galactose-deficient IgA1 (the Tn antigen GalNAcα1-Ser/Thr) in the glomerular mesangium. However, the nature of such circulating immune complexes is largely undefined. Using biochemical approaches, we found that novel anti-Tn circulating immune complexes (anti-Tn CICs) containing IgA1 with predominantly IgM and some IgG. Our blue native agarose acrylamide gel electrophoresis (BN-APAGE) analysis showed anti-Tn CICs resolving at large macromolecular complexes of ~1.2 MDa to several MDa sizes. Additionally, we found that in IgAN patient sera, there are significantly elevated levels of anti-Tn CICs, and our preparation of purified anti-Tn CICs contains a higher level of complement C3, compared to healthy individuals. BN-APAGE analysis showed that anti-Tn CICs can be dissociated with small glycomimetic compounds which mimic the Tn antigen of Tn(+)IgA1, releasing

polymeric IgA1 from the anti-Tn CICs purified from IgAN patients. Serum analysis of IgAN patients showed dose-dependent proliferation of human renal mesangial cells compared to healthy control; such proliferative nature could be specifically reduced by immunodepleting anti-Tn CICs from the IgAN serum and can be restored by the addition of the purified anti-Tn CICs in a dose-dependent manner. We found that these anti-Tn CICs bind to the surface of the human renal mesangial cells. The Tn antigen-based glycomimetic compound can significantly inhibit the proliferative activity of anti-Tn CICs of IgAN patients. Our results define novel elevated levels of circulating immune complexes of IgAN, containing galactose-deficient IgA1, and provide a potential noninvasive diagnosis and treatment option for IgAN, as no specific drug treatments for this disease are currently available.

(P146) Glycans in neural wiring/rewiring

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It is emerging that glycans play important roles in neural wiring/rewiring. We found that the extracellular chondroitin sulfate (CS) induced in neural injuries monomerizes and activates the receptor-type tyrosine phosphatase PTPs on axon terminals, where it disrupts autophagy flux and consequently inhibits axon regeneration. In contrast, heparan sulfate (HS) oligomerizes and inactivates PTPσ and promotes axon regeneration. These opposite effects of CS and HS are due to the difference of functional domains of these glycans in the brain (1). The glycan-PTPs axis may play not only pathologic but also physiologic roles. Indeed, the combination of HS/CS and PTPs regulates steps of synapse formation (2, 3, unpublished data). We also found that the receptor-type tyrosine kinase ALK is expressed in the central nervous system and promotes axon extension. Using the method proximity-dependent biotin identification, we determined that PTPs and ALK have several common substrates. This suggests that the crosstalk between these tyrosine phosphatase and kinase through glycans may play a role in neural functions. Finally, I would like to address another important issue. When considering life science, it is no exaggeration to say that the overwhelming lack of information on glycans, one of the three major life chains, compared to genomes and proteins, has limited our ability to solve life. It is time for us to develop a research infrastructure that will boost glycan information to the level of genome and protein information and make the big data available to researchers in various fields of life science. (1) Sakamoto K et al. Glycan sulfation patterns define autophagy flux at axon tip via PTPRσ-cortactin axis. Nat Chem Biol. 2019 Jul;15(7):699-709. (2)Tran AP et al. Regulation of autophagy by inhibitory CSPG interactions with receptor PTPσ and its impact on plasticity and regeneration after spinal cord injury. Exp Neurol. 2020 Jun;328:113276. (3) Sakamoto K et al. Type IIa RPTPs and Glycans: Roles in Axon Regeneration and Synaptogenesis. Int J Mol Sci. 2021 May 24;22(11):5524

(P147) The highly diversified lectome of the softshell clam (*Mya arenaria*): Recognition of sympatric *Perkinsus* parasite species by galectins and R-type lectins

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Invertebrates display effective innate immunity for defense against microbial infection with a diversified repertoire of soluble and cell-associated lectins mediate binding interactions with potential pathogens. Among them, the highly conserved galectins are key to multiple biological functions, including pathogen recognition and regulation of immune responses. We previously showed that the galectins CvGal1 and CvGal2 from the eastern oyster (*Crassostrea virginica*) preferentially bind ABH blood group oligosaccharides and play a significant role in infections by the parasite *Perkinsus marinus*. CvGals bind to "self" glycans on the hemocyte surface, and recognize potential microbial pathogens and unicellular algae. They preferentially bind to *P. marinus* trophozoites, but not to *P. chesapeaki*, a sympatric species mostly prevalent in the soft-shell clam *Mya arenaria*. We recently isolated a novel galectin from the softshell clam (MaGal1) that strongly cross-reacts with anti-CvGals antibodies, and preferentially binds to Gal(α1-3/4) Gal(β1-3/4)GlcNAc. Consistently, it preferentially binds to asialofetuin over PSM, the preferred ligand for CvGals. We hypothesize that differential recognition of *Perkinsus s*pecies by oyster and clam lectins facilitate parasite entry and infectivity by carbohydrate-based parasite mimicry in a host-preferential manner, and are responsible for their relative prevalence and pathogenicity in oyster and clam species. To extend our knowledge about the diversity of the lectin

repertoire of the bivalves, we performed a transcriptomic analysis on *M. arenaria* by RNAseq: 167 unigenes were tentatively identified as lectin-like proteins, including C-type lectins, galectins, and R-type lectins. BLAST analysis revealed a tandem-repeat galectin (MaGal1), with high homology to Manila clam (*Venerupis philippinarum*) galectin, a galectin-3-like galectin (MaGal3), and a galectin-9-like galectin (MaGal9). Analysis of both the clam and oyster transcriptomes and genomes with the LectomeXplore bioinformatic tool on the UniLectin database identified several additional lectin families present in the two bivalve species that had not been detected by mining the transcriptome. By proteomic and BLAST analyses we also identified a lactose binding lectin (MaRTL) with high homology to the mussel R-type lectin (MytiLec3). Current studies are aimed at the structural basis for the differential recognition *Perkinsus* species by clam lectins MaGal1, MaGal3, MaGal9 and MaRTL, and the oyster galectins CvGal1 and CvGal2. [Supported by NSF grant IOS-1656720 to GRV. We are grateful to Dr. R. D. Cummings and Dr. J. Heimburg-Molinaro, NCFG, for glycan microarray analysis].

(P148) Peering into the Donor Binding Site of Phosphoglycosyl Transferases Belonging to the "Stealth Family"

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The bacterial capsule protects bacteria from host immune systems and is an important virulence factor. It is composed of a variety of monosaccharides joined through glycosidic or phosphodiester linkages. Synthesis of capsule through formation of these chemical bonds is catalyzed by glycosyl and phosphoglycosyl transferases, respectively. While glycosyl transferases are relatively well understood, mechanistic and structural information is lacking in phosphoglycosyl transferases. Studying the mechanism and active site interactions of enzymes from the 'stealth family' can potentially translate into new vaccine production technologies and antibacterial treatments. CsxA from *Neisseria meningitidis* Serogroup X is a phosphoglycosyltransferase from the stealth family, that catalyzes the synthesis of $\alpha(1\rightarrow4)$-linked *N*-acetylglucosamine (GlcNAc)-1-phosphate homopolymer using UDP GlcNAc as the donor substrate. We measured the binding of sugar and base modified UDP GlcNAc analogs, using microscale thermophoresis (MST) to understand enzyme substrate interactions at the active site of CsxA. Using C-2 and C-4 modified UDP GlcNAc analogs, we were able to conclude that the presence of an acyl group at C-2 and a hydrogen bond donating hydroxyl group at C-4 are important for donor substrate binding. In addition to its role as an acceptor in capsular polysaccharide chain elongation, the C-4 hydroxyl group is important for donor substrate binding and recognition. By analyzing the binding of C-5' halogen substitutions we hypothesize that the uracil group is held in place by an aromatic stacking interaction. Substitution of a bulkier 5-formylthien-2-yl group at the C-5' position resulted in an inhibitor that binds 3-fold tighter to CsxA than its natural substrate. Binding studies using constructs of CsxA with mutations at a highly conserved tryptophan, indicated that the presence of an aromatic side chain at that position is essential for substrate binding. Considering lack of structural data for the stealth family of phosphoglycosyl tranferases, this information adds to our understanding of these enzymes.

(P149) Ultraviolet photodissociation provides validation of additional glycosites on *O*-glycopeptides cleaved with O-glycoprotease

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Introduction: Despite the prevalence of glycosylated proteins – approximately 50% of all proteins contain are glycosylated – O-glycans are far less studied than *N*-glycans due to their varied core structures and lack of a universal enzyme to aid in their analysis. While an enzyme to achieve cleavage between the peptide backbone and O-glycan is still elusive, the discovery of new proteases for the specific generation of O-glycopeptides are a promising step forward to determine previously overlooked glycosites and their various glycoforms. Immunomodulating metalloprotease (IMPa) is one such protease that cleaves a peptide backbone N-terminal to O-glycosylated serines and threonines, resulting in O-glycopeptides with a terminal O-glycan. However, if a proximal glycosylation occurs, the protease is unlikely to cleave at the second O-glycosite. Thus, a way to localize and characterize additional glycosites is necessary to fully study the impact of IMPa on O-glycopeptide analysis. Ultraviolet photodissociation (UVPD) has proven useful in the study of post-translational modifications and can facilitate the characterization of glycan compositions and localization of glycosylation sites. The combination of UVPD with a O-glycoprotease that reliably cleaves at O-glycosites offers a promising new strategy for characterization of glycopeptides and localization of glycosites.

Methods: Synthetic glycopeptides containing two adjacent glycosites were reconstituted in 1:1 water:methanol with 0.1% formic acid for optimization of LC-MS/MS analysis. Intact glycoproteins were digested with 1 unit of O-Glycoprotease (New England Biolabs) and incubated for 5 h at 37 °C. O-glycopeptides were separated from undigested glycoprotein using 30 kDa

molecular weight cut-off filters and dried down in a SpeedVac. Peptides were reconstituted in water and analyzed by LC-MS/MS using a Dionex 3000 UltiMate Nanoflow HPLC (Thermo Scientific) coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific).

Results: Model glycopeptides containing a single N-acetylgalactosamine were subject to 1-8 pulses of 193 nm UVPD with energies between 0.5 and 4 mJ. Optimal conditions were determined by the abundances of bracketing ions between adjacent glycosites. Glycopeptides were selected using a HCD-triggered UVPD method; oxonium ions common for O-glycans comprised the inclusion list to trigger UVPD of the glycopeptides. UVPD allowed localization of glycosites based on production of fragment ions retain the O-glycan.

Conclusions: The ability of UVPD to provide information on the glycan and localize the glycosite as well serves as an excellent tool for the exploration of O-glycopeptides generated from a specific O-glycoprotease.

(P150) Protein *O*-Glucosyltransferases 2 and 3 are essential for optimal microfibril formation and elastin deposition in mouse lung

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Epidermal Growth Factor-like (EGF) repeats are small protein domains with six cysteines forming three disulfide bonds, found in hundreds of cell-surface and secreted proteins in metazoans. Over the past 22 years, four different types of *O*-glycosylation have been detected on EGF repeats, including *O*-glucose (at 2 different sites), *O*-fucose, and *O*-GlcNAc. Five different Protein *O*-glycosyltransferases are responsible for addition of these sugars to distinct consensus sites in EGF repeats. All of these enzymes are localized to the endoplasmic reticulum and only modify properly folded EGF repeats. They appear to have evolved at the same time that proteins containing EGF repeats appeared in metazoan lineages. Mutations in *POGLUT1*, *POFUT1*, and *EOGT* result in congenital disorders of glycosylation in humans affecting the Notch pathway, and knockout of these enzymes in mice results in Notch-related phenotypes. Many *in vitro* studies reveal that these modifications are essential for Notch trafficking and function. More recently we identified POGLUT2 and POGLUT3, homologs of POGLUT1 which add *O*-glucose to a distinct site from POGLUT1 in EGF repeats. While POGLUT2 and 3 modify a few EGF repeats in NOTCH1 and NOTCH3, elimination of those sites has a small effect on Notch activity. In contrast, Fibrillins (FBNs) and Latent TGFβ Binding Proteins (LTBPs) appear to be the major targets of these enzymes. FBN1 and 2 are the major structural proteins forming microfibrils in the Extracellular Matrix. They serve as the scaffold for Elastin deposition in elastic tissues. LTBPs bind to FBNs and regulate TGFβ signaling. Mutations in *FBN1* result in Marfan Syndrome and acromelic dysplasias. FBN1 and 2 have 47 EGF repeats, and over 50% of them are modified by POGLUT2 and/or POGLUT3. Double knockout of *POGLUT2/3* in HEK293T cells causes loss of *O*-glucose modifications and reduced secretion of FBNs, suggesting that this modification could play a role in folding and trafficking of these proteins. We have generated mice lacking each of these enzymes. Single knockouts of either *Poglut2* or *Poglut3* are viable and fertile with no observable phenotypes. The majority of double knockouts die soon after birth with phenotypes similar to those seen in *Fbn* and *Ltbp*-null mice, including reduced levels of FBN1/2 and elastin in lung tissues and reduced TGFβ signaling. These results suggest that in contrast to POGLUT1, POFUT1 and EOGT, the major biological targets of POGLUT2 and 3 are FBNs and LTBPs. This work was supported by NIH grants GM061126 and HL161094.

(P151) Establishment of Blood Glycosidase Activities and their Excursions in Sepsis

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Glycosidases are hydrolytic enzymes studied principally in the context of intracellular catabolism within the lysosome. Therefore, glycosidase activities are classically measured in experimentally acidified assay conditions reflecting their low pH optima. However, glycosidases are also present in the bloodstream where they may retain sufficient activity to participate in functions including the regulation of glycoprotein half-lives, proteostasis, and disease pathogenesis. We have herein established at physiological pH 7.4 in blood plasma and sera the normal ranges of four major glycosidase activities essential for blood glycoprotein remodeling in healthy mice and humans. These activities included b-galactosidase, b-N-acetylglucosaminidase, a-mannosidase, and a-fucosidase. We have identified their origins to include the mammalian genes *Glb1*, *HexB*, *Man2a1*, and *FucA1*. In experimental sepsis, excursions of glycosidase activities occurred with differences in host responses to discrete bacterial

pathogens. Among similar excursions in human sepsis, the elevation of b-galactosidase activity was a prognostic indicator of increased likelihood of patient death.

(P152) Using GlycoSim to simulate O-glycosylation of mouse ES cells

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GlycoSim is a Web tool to enable users to finely simulate glycosylation *in silico*. We have recently incorporated a database of reaction parameters into GlycoSim to allow users to more easily input the specific details required for such simulation. New supportive tools such as parameter estimation and sensitivity analysis has also been implemented, as well as a new module for retrieval of models from the BioModels database[1]. In this work, we show how we were able to use GlycoSim to simulate the O-glycosylation of mouse ES cells[2] and exactly match experimental results. We also attempted to simulation Notch glycosylation[3] patterns by retrieving a Notch signaling pathway from the BioModels database, and then incorporating glycosylation reactions onto specific EGF domains. We hope that users will find this tool useful to emulate knock-out and knock-down experiments *in silico*, to help not only with understanding glycosylation but also assisting in planning future experiments. GlycoSim and the signaling module GSS are available on the new RINGS server at https://glycosim.rings.glycoinfo.org/.

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(P153) New players essential for recycling of early Golgi glycosylation enzymes

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Maintenance of the correct subcellular localization of enzymes is critical for cellular homeostasis. Glycosylation is the most abundant and diverse post-translational protein modification mediated by sequential action of transmembrane glycosyltransferase enzymes appropriately maintained in specific Golgi compartments. Using yeast genetics and fluorescence microscopy, we have uncovered the integral membrane protein in yeast, Erd1, as a key facilitator of Golgi glycosyltransferase recycling by directly interacting with both the Golgi enzymes and the cytosolic receptor, Vps74. Loss of Erd1 function results in mislocalization of Golgi enzymes to the vacuole/lysosome. We present evidence that Erd1 forms an integral part of the recycling machinery and ensures productive recycling of several early Golgi enzymes. Our work provides new insights on how the localization of Golgi glycosyltransferases is spatially and temporally regulated and is finely tuned to the cues of Golgi maturation.

(P154) A human lectin array for screening of microbial ligands

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A human lectin array has been developed to probe the interactions of innate immune receptors with pathogenic and commensal micro-organisms. Following the successful introduction of a lectin array containing all of the cow C-type carbohydrate-recognition domains (CRDs), a first version of a human array described here contains the C-type CRDs as well as CRDs from a selection of other classes of sugar-binding receptors, including galectins, siglecs and ficolins. The array is constructed with CRDs modified with single-site biotin tags, ensuring that the sugar-binding sites in CRDs are displayed on a streptavidin-coated surface in a defined orientation and are accessible to the surfaces of microbes. In addition to previously documented protocols for binding of micro-organisms that express green-fluorescent protein or are chemically labelled, unlabelled bacteria bound to the array can be detected by counter-staining with DNA stains.

Preliminary results demonstrate that: (1) A common approach can be used for expression and display of CRDs from all of the different structural categories of glycan-binding receptors, allowing comparisons across lectin families. (2) Receptors in some groups, such as the galectins or the mannose-binding C-type lectins DC-SIGN, langerin, and the mannose receptor, bind many of the same micro-organisms, but in each case there are some micro-organisms that bind only to a subset of the group. (3) Lipopolysaccharides, pilus glycoproteins, wall polysaccharides and capsular polysaccharides are all potential bacterial ligands

for human receptors, but some of these targets can be masked in intact micro-organisms. (4) Some receptors usually associated with binding of endogenous mammalian glycans, such as the asialoglycoprotein receptor, also bind to bacteria. (5) The array provides an unbiased screen for sugar ligands that interact with receptors and many show binding not anticipated from earlier studies. For example, some of the galectins bind with high affinity to bacterial glycans that lack lactose or N-acetyllactosamine. Similarly, LSECtin binds well to yeast and bacteria that do not contain the GlcNAcβ1-2Man epitope identified as the common binding motif in glycan array screening of this receptor. (6) When binding is not readily explained based on known structures on micro-organisms, the biotin-tagged receptors can be used as tools for identification and characterization of target ligands.

These results demonstrate the utility of the human lectin array, providing a unique overview of the interactions of multiple classes of glycan-binding proteins in the innate immune system with different types of micro-organisms.

(P155) Neu5Ac and Neu5,9Ac2 in Human Plasma: Potential Biomarkers of Cardiovascular Disease

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Cardiovascular disease (CVD) poses an extreme global healthcare burden, accounting for 32% of all deaths in 2019.[1] CVD is characterized by increased levels of inflammation which has been associated with a rise in the concentration of *N*-acetyl neuraminic acid (Neu5Ac) in blood.[2] 9-*O*-Acetyl-*N*-acetyl-neuraminic acid (Neu5,9Ac$_2$) has been of interest as a biomarker for diseases such as breast cancer but has not been studied in the case of CVD.[3] Neu5Ac and Neu5,9Ac$_2$ concentrations were determined by quantitative analysis using ultra-high-performance liquid chromatography in plasma samples obtained from both patients with CVD and healthy controls.[4] Mean concentrations of Neu5Ac and Neu5,9Ac$_2$ were significantly elevated between the two sample groups (Neu5Ac: $P < 0.001$; Neu5,9Ac2: $P < 0.04$). Receiver operator curve analysis (ROC) further revealed the predictive power of the two markers (Neu5Ac AUC: 0.95; Neu5,9Ac2 AUC: 0.84). A combined Neu5Ac/Neu5,9Ac$_2$ marker exhibited an AUC of 0.96. The sensitivity (true positive) and specificity (true negative) of each marker was subsequently assessed. Neu5Ac and Neu5,9Ac$_2$ both showed good specificity (Neu5Ac: 0.9; Neu5,9Ac$_2$: 0.9), but only Neu5Ac showed adequate sensitivity (Neu5Ac: 0.88; Neu5,9Ac$_2$: 0.5). The combined Neu5Ac/Neu5,9Ac$_2$ marker offered similar sensitivity (0.88) to Neu5Ac but a much higher specificity (1.0) indicating a zero false positive rate. Overall, Neu5Ac appears to have good discriminatory power for CVD. Combining the two markers together may offer a better biomarker than either of the markers individually. Further analysis was undertaken to determine the N-glycan profile, percentage galatosylation of N-glycans and c-reactive protein concentration of each sample. ROC analysis was performed and AUC values were calculated for these analytes and compared with the ROC data for Neu5Ac and Neu5,9Ac$_2$. The samples were also analysed via a novel column-free prefractionation system, which uses nanoparticles as an enrichment tool.[5,6] This allowed for the extraction of fibrinogen, which is a known inflammatory marker, from the plasma and subsequent analysis of fibrinogen derived glycans. Following this, analysis was performed to determine any increase in the concentrations of these glycans between CVD cases and healthy controls.

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(P156) Age-associated changes in brain extracellular matrix plasticity across species

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Brain plasticity, which can be defined as the brain's ability to reorganize its connections in response to biological stimuli, is driven by changes in the extracellular matrix chondroitin sulfate glycosaminoglycan (CS-GAG) sulfation patterning. Specifically, the CS-C (6S) isomer promotes neurocircuit plasticity by forming a soft matrix pallet and altering extracellular protein-glycan

binding interactions involved in circuit remodeling. Recent studies demonstrate a steady decrease in CS-C (6S) isomer throughout natural aging in mice, which is hypothesized to underlie age-related decline in cognitive function. To further explore whether these age-related changes in brain extracellular matrix plasticity in rodents also occur in humans, we analyzed the relative abundance of CS isomers in n=54 hippocampal and cortical samples in human brain tissue (age: newborn – 95 y, sex: 50%M). As observed mice, human hippocampus and cortex exhibit the highest abundance of neuroplasticity CS-C (6S) isomer after birth (<1 month age; 19.8% HIP; 11.5% CTX; *p=0.08*) compared to the lowest abundance in early adulthood (20-29 y; 5.5% HIP; 1.5% CTX; *p=0.01*). Surprisingly, the abundance of CS-C (6S) steadily increases throughout life after 20 y of age (HIP, R^2=0.44, *p=0.0001*; CTX, R^2=0.24, *p=0.0096*), with the hippocampus exhibiting a faster rate of CS-C (6S) accumulation compared to cortex (*p=0.0439*). The steady increase in CS-C (6S) isomer peaks a second time in elderly-adults (80-99 y; 9.7% HIP; 3.1% CTX; *p=0.0001*). This biphasic modeling of extracellular matrix neuroplasticity in human brain is in stark contrast to the age-related changes observed in mice (age: 10 d – 2 y; sex: 50%M), which shows the highest abundance of hippocampal CS-C (6S) at 10 d (38.8%) compared to 1 and 2 y of age (5.1% 1 y; 5.1% 2 y; *p=0.83*). Overall, these results suggest that the age-associated increase in brain extracellular matrix CS-C (6S) isomer in human brain tissue does not recapitulate the age-related decline in CS-C (6S) observed in mouse brain, and that further research is required to identify alternative models to study natural aging and other age-related extracellular matrix diseases in humans.

(P157) GlyGen Glycan Structure Archive: An archival repository for enhanced stability and availability of glycan records

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Technological advances in the field of glycoscience have led to a rapidly growing volume of glycan and glycosylation data, resulting in a need for a primary database with archival functionality that not only standardizes the process of reporting glycan structures but also preserves data provenance. While a number of international resources and repositories have emerged to facilitate harmonization and integration of glycan data, the glycoscience community lacks an archive that maintains individual primary records of submitted glycan entries for historical record keeping and evidence tracking similar to what GenBank does for nucleotide sequences. The GlyGen project has developed a prototype Glycan Structure Archive (GSA) to address the need for an evidence-based archival repository. The GSA maintains glycan records consisting of a stable record identifier (GSA ID), structure sequence (WURCS or GlycoCT), GlyTouCan accession, source and experimental information, associated glycoconjugate information, publications, and mapping to other databases (GlyGen, ChEBI, PubChem, GlyTouCan), whenever possible. Each unique GSA ID allows the same glycan structure to be submitted more than once if contributed by different databases or researchers or if present in different biological contexts, such as different sites of a protein. Initially, the GSA prototype was populated with glycan records sourced from existing public databases such as GlyTouCan, GlyGen, and PubChem. However, a web portal allows users to browse and download existing glycan records, make new submissions, and update their own submissions. Submissions undergo quality checks for syntactical correctness, metadata completion, valid data formats, and validation by the GlycoTree framework, which flags structures that are inconsistent with human curated bio-enzymatic rules. This feature allows users to check their entry for possible errors but does not block the submission of novel glycans that might indicate new biosynthetic possibilities. All records are publicly available and can be used by other glycan and chemical centric resources and researchers. The Glycan Structure Archive will enhance stability and promote cooperation among the international glycoscience community. This work was supported by NIH grant 3U01GM125267-05S1.

(P158) Receptor protein tyrosine phosphatase is required for sensory axon wiring and regulated by O-mannosylation in Drosophila

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Protein O-mannosylation is a type of post-translational modification that affects the development of muscular and nervous systems in animals. Previous studies discovered that Protein O-mannosyltransferases 1 and 2 (POMT1/2) play a role in correct neuronal wiring in the development of the nervous system in *Drosophila*. This subfamily of enzymes mediates the addition of O-linked mannose to Ser/Thr residues of proteins in the endoplasmic reticulum. Mutations in POMT1/2 result in severe congenital muscular dystrophies associated with neuromuscular abnormalities caused by defective glycosylation of α-Dystroglycan (α-Dg). Although these defects are known to underpin muscle degeneration, the relationship between the neurological phenotypes

and Dg remains poorly understood and other substrates of POMT1/2 remain largely uncharacterized. Our experiments have shown that Dg alone does not account for body torsion and abdomen rotation phenotypes in *Drosophila*, indicating the involvement of other substrates in the POMT-mediated pathway. To shed light on new functional targets of POMTs in the nervous system, we used *Drosophila* to reveal genes that interact with *POMT1/2* and produce similar neurological phenotypes. We identified *Receptor Protein Tyrosine Phosphatase 69D* (*PTP69D*) as a gene interacting with *POMT1/2* in producing the abdomen rotation phenotype. Using a variety of genetic approaches, we revealed that *PTP69D* is required for sensory axon wiring in the ventral ganglion of *Drosophila* larvae. *PTP69D* and *POMT1/2* show complex interactions, showing synergistic or antagonistic effects on axon wiring phenotypes when these genes are downregulated in sensory neurons only or globally, respectively. Using glycoproteomic approaches, we characterized the glycosylation of a PTP69D transgenic construct expressed in *Drosophila* genetic strains with different levels of *POMT1/2* activity. We found that the PTP69D construct carries multiple O-linked hexose modifications when expressed in wild-type or *POMT1/2* over-expression backgrounds, but these modifications were absent in a *POMT1/2* mutant background, suggesting that PTP69D is a substrate of O-mannosylation by POMT1/2. Taken together, our results indicate that PTP69D is required for axon connectivity mediated by POMT1/2 O-mannosylation. This mechanism is potentially conserved in mammals and may shed light on the involvement of POMT1/2 in human pathologies.
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(P159) Sialylation Regulates Binding Interactions Between Human Plasma Fibronectin and Galectin-3

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The extracellular matrix (ECM) is a multi-component, tissue-specific structure comprised of proteins, proteoglycans, and glycosaminoglycans. Many ECM proteins are glycosylated (i.e., "glycoproteins"); however, little is presently known about the effects of glycosylation on ECM glycoprotein form and function. Recent glycomics studies indicate that the sialylation profile of ECM glycoproteins, like fibronectin, can differ based on the source, the developmental stage of the organism, or disease state, with desialylation leading to increases in glycoproteins with terminal N-Acetyllactosamine (LacNAc) groups. Galectin-3 is a LacNAc-binding protein that has been suggested to contribute to cell-matrix adhesion, while elevated serum levels of both fibronectin and galectin-3 are associated with chronic inflammation. However, there are conflicting perspectives on whether galectin-3 and fibronectin interact directly. Using a GFP-Gal3 fluorescent reporter developed by our lab,[1,2] we demonstrate that galectin-3 binds to human plasma fibronectin. Additionally, pre-treatment of human plasma fibronectin with sialidase increases the extent of galectin-3 binding. The dissociation constant of GFP-Gal3 for fibronectin did not change after sialidase treatment, suggesting that removal of sialic acids does not expose glycans with higher monovalent (1:1) binding affinity for galectin-3, but instead only increases the number of galectin-3-binding glycans. We have previously shown that GFP-Gal3 does not form oligomers upon glycan binding,[1,2] in contrast with wild-type galectin-3. Collectively these data demonstrate that binding can occur between human plasma fibronectin and galectin-3, but that this binding depends on the sialylation profile of fibronectin. Source- or disease-dependent changes in sialylation state may explain the conflicting roles of galectin-3:fibronectin interactions in cell-ECM adhesion.

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(P160) Blood Group A Enhances SARS-CoV-2 Infection

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Severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) continues to infect millions of individuals worldwide. However, not all individuals are equally susceptible to infection. Many studies have demonstrated that the first polymorphism

described in the human population, ABO(H) blood group antigens, are associated with increased risk of SARS-CoV-2 infection. While conflicting data exist, the most common findings suggest that individuals with blood group A exhibit an increased risk of infection when compared to blood group O individuals. However, the mechanism whereby ABO(H) blood group status influences SARS-CoV-2 infection has remained unknown. The receptor binding domain (RBD) of SARS-CoV-2, which facilitates host cell engagement, bears significant similarity to galectins, an ancient family of carbohydrate binding proteins previously shown to recognize ABO(H) blood group antigens. Sequence analysis of galectins demonstrates that several galectins possess up to 11% sequence identity with the RBDs of SARS-CoV-2 WT (Wuhan-Hu-1) and variants (Delta and Omicron). Each RBD (Wuhan, Delta and Omicron) exhibited glycan binding specificity that overlapped with distinct members of the galectin family, with the highest similarity observed toward the C terminal domain of galectin-4 (Gal-4C). Direct comparison of binding toward ABO(H) antigens demonstrated that each RBD and Gal-4C displayed high specificity for the type 1 blood group A structure uniquely found on respiratory epithelial cells when compared to other blood group antigens. To specifically examine the impact of blood group A on SARS-CoV-2 infection, CHO cells were engineered to express ACE2 and the type 1 blood group A or H antigen (blood group O) normally found on respiratory epithelial cells. Consistent with the increased binding of the RBD to blood group A on the glycan microarray, enhanced binding was observed by each RBD toward blood group A expressing cells. Blood group A cells were also significantly more likely to be infected with Wuhan-Hu-1, Delta and Omicron variants of SARS-CoV-2 when compared to blood group O cells, while pre-incubation of cells with Gal-4C specifically inhibited infection of blood group A expressing cells, while failing to impact infection of blood group O cells. These results demonstrate a direct effect of blood group A on viral infection and suggest that blood group A individuals may exhibit an increased susceptibility to SARS-CoV-2 as a result of direct engagement of the blood group A antigen. However, variations in ACE2 levels, blood group A expression and many other factors likely influence the overall risk of SARS-CoV-2 infection following exposure in a given individual. The present results provide one mechanism whereby blood group A itself may directly influence SARS-CoV-2 infectious risk.

(P161) Hyperactive innate immune response and altered metabolism in a Drosophila model of NGLY1 deficiency

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Dysregulation of intestinal innate immune response and metabolism are implicated in chronic inflammatory intestinal diseases associated with high morbidity in humans. However, the mechanisms underlying aberrant innate immunity and metabolism and the mechanistic links between these two processes are not well understood. Using *Drosophila* as a model, here we establish that loss of the deglycosylation enzyme *N*-glycanase 1 (NGLY1; Pngl in *Drosophila*) leads to a hyperactive innate immune response and increased lipid catabolism in the larval intestine, which together contribute to animal lethality. The increased innate immune response in *Pngl* mutants is primarily driven by overactivation of the forkhead box O family transcription factor Foxo in midgut enterocytes and the fat body, which is equivalent to liver and adipose tissues in mammals. Our data suggest that decreased insulin signaling and increased JNK pathway activity both contribute to Foxo overactivation. *Pngl* mutant larvae show evidence for starvation and increased lipid catabolism. Importantly, raising *Pngl* mutant larvae on an isocaloric high-fat diet results in a partial rescue of lethality and a full rescue of developmental delay, strongly suggesting that a shortage of energy supply contributes to developmental delay and lethality in these animals. *Pngl* mutant larvae exhibit impaired gut barrier function, which in turn contributes to starvation, Foxo activation, and subsequent hyperactivation of innate immune genes. Taken together, our data indicate that NGLY1 functions in several cell types in the *Drosophila* larval intestine to maintain normal gut barrier, prevent innate immune hyperactivation, and ensure metabolism homeostasis during *Drosophila* development.

(P162) Post-translational glycosylation of polypeptide tags for modification of protein assembly and receptor targeting

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Protein glycosylation can affect all levels of protein complexity, from assembly to functions such as binding to lectin receptors. However, difficulties exist in precisely modifying carbohydrate type, density, and valency to govern these effects. Recombinant polypeptide tags allow user-defined glycosylation to engineer protein function. We have designed two recombinant tags that consist of either a repeating *Actinobacillus plueropneumoniae* N-glycosyltransferase (ApNGT) consensus sequence or polypeptide N-acetylgalactosaminyltransferase 2 (ppGalNAcT2) consensus sequence, fused onto the N-terminus of superfolder

green fluorescent protein for fluorimetric detection (N-glycotag-sfGFP, O-glycotag-sfGFP). N-glycotag-sfGFP and O-glycotag-sfGFP could be efficiently enzymatically glycosylated with ApNGT and UDP-Glucose (Glu) or ppGalNAcT2 and UDP-N-acetylgalactosamine (GalNAc), respectively, to make Glu-N-glycotag-sfGFP or GalNAc-O-glycotag-sfGFP. N-glycotag-sfGFP displays an upper critical solution temperature (UCST), undergoing a sol-gel transition at 4°C, and transitioning back to liquid state above 50°C. Glucose modification disrupted the UCST, and Glu-N-glycotag-sfGFP retained miscibility through 4°C-60°C. These data demonstrate the ability of glycosylation to influence phase behavior, and potential uses to engineer glycosylation states for precise control of the UCST. For lectin receptor engagement, we investigated liver cell uptake of GalNAc-O-glycotag-sfGFP using HepG2 cells as an *in vitro* model for human hepatocytes. HepG2 cells were cultured with either O-glycotag-sfGFP, GalNAc-O-glycotag-sfGFP, or untagged sfGFP. HepG2 cells preferentially associated with GalNAc-O-glycotag-sfGFP quantified via microscopy and flow cytometry. We next investigated whether this association was mediated by cell surface carbohydrate receptors specific for GalNAc or non-specific interactions. HepG2 cells were cultured with soluble GalNAc, which provided competitive inhibition for GalNAc specific receptors, or L-rhamnose, an irrelevant carbohydrate, before being cultured with GalNAc-O-glycotag-sfGFP. Cells treated with L-rhamnose or no soluble sugar had significantly higher sfGFP signal than cells treated with GalNAc. This data supports the use of the GalNAc-O-glycotag as a recombinant multivalent carbohydrate tag for hepatocyte engagement through GalNAc specific receptors.

(P163) TMTC O-mannosyltransferases are required for the development of neural connectivity in Drosophila

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Recently discovered noncanonical protein O-mannosyltransferases of TMTC subfamily (Transmembrane O-Mannosyl transferases Targeting Cadherins) were shown to add O-linked mannose to cadherins, protocadherins, and some other proteins, however, the biological functions of TMTC-mediated O-mannosylation remain poorly understood. Cadherins, a large family of transmembrane glycoproteins, are crucial for cell adhesion. They play many essential roles in metazoans, regulating neural development, epithelial maintenance, and affecting tumor suppression. Although O-mannose has been found on cadherins within important functional domains, the *in vivo* function of these modifications remains unclear. Notably, *TMTC* mutations were found to be associated with brain malformations and neurological disorders, which suggests that TMTCs play important roles in nervous system development, possibly by affecting the function of neural cadherins.

In this study, we focus mainly on *Drosophila* TMTC1 & TMTC2 and their functions in the nervous system. Previously, our analyses revealed that these highly homologous genes are expressed in an overlapping pattern restricted to the nervous system. Simultaneous inactivation of these genes is semilethal, causing lethality during larval and pupal stages. Our initial analyses revealed that *TMTC1* and *2* mutants have defects in neural connectivity, affecting the wiring of sensory axon termini and the morphology of main axon tracts in the larval brain. Rescue experiments suggested that these enzymes are partially redundant, while also having non-overlapping functions. Notably, *N-Cadherin* mutants show axonal defects similar to those found in *TMTC* mutants, suggesting that TMTC-mediated O-mannosylation is important for N-Cadherin functions required for proper neuroarchitecture in the developing brain. This conclusion was further supported by our glycoproteomic analyses, which indicated that N-Cadherin is a substrate for O-mannosylation in *Drosophila*, and that this modification is potentially dependent on the activity of TMTC1/2. Intriguingly, protein sequence alignments revealed conservation of O-mannosylation sites between *Drosophila* and human cadherins, suggesting that the function of cadherin O-mannosylation is evolutionarily conserved. Finally, we establish HEK293 cell lines stably expressing *Drosophila* TMTCs and probe their glycosylation capacity in the context of the human proteome. We demonstrate that individual Drosophila TMTCs are functionally conserved and capable of inducing O-mannosylation of multiple human cadherins and protocadherins, including N-cadherin. Taken together, our results contribute to understanding the function of protein O-mannosylation in nervous system development and the function of cadherins in *Drosophila*, having potential implications for uncovering similar mechanisms in mammals, including humans.
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(P164) Highly ordered polymeric structure assemblies of endoglycosidases from the gut microbiome

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A critical variable that dictates the composition and physiology of the microbiota is the influx of complex carbohydrates and glycans into the intestine, mostly from diet and host mucosal secretions. The initial step of glycan processing by human

gut bacteria is the liberation of glycans from glycoproteins which is carried out by endoglycosidases (ENGases). ENGases are commonly co-localized in discrete clusters known as polysaccharide utilization loci (PULs), dedicated to the liberation, capture, importation, and processing of a certain glycan type. These glycans are then captured on the bacterial cell surface and further processed to be used as an energy source by the microbe. In this study, we found that the High-Mannose specific endoglycosidase BT3987, belonging to GH18 family from *Bacteroides thetaiotaomicron*, was able to form temperature, pH and salt-dependent soluble polymeric assemblies. Size exclusion chromatography (SEC) and dynamic light scattering (DLS) were used to determine particle size and negative staining transmission electron microscopy (NS-TEM) was used to evaluate shape and obtain low resolution structures of polymers. DLS analysis as a function of temperature revealed the formation of BT3987 nanoparticles at temperatures higher than 50° C. These polymers were highly stable- their hydrodynamic size did not change over the course of months nor did re-heating the nanoparticles have an impact on size. The polymeric assemblies were separated by SEC and filaments of specific size were assayed for endoglycosidase activity measurements by LC-MS method. These polymers were able to specifically deglycosylate HMNG containing glycoproteins, partially retaining catalytic activity of the monomers. NS-TEM analysis showed linear and flexible polymeric filaments of approximately 50 nm in length and 10 nm of width after heating at 70° C for 5 min. We obtained a low-resolution structure (around 20 Å) of the ordered filament containing four protomers assembled. In addition, we tested different conditions of temperature, salt, pH, detergent and reducing agent, and it was identified the formation of polymers in some experimental conditions within the physiological range, suggesting that these polymers could potentially form on the cell surface in some environmental conditions encountered by *B. thetaiotaomicron*. These *in vitro* findings suggest that this enzyme may polymerize on the bacterial cell surface to act as nanomachines to enhance the capture and hydrolysis of select glycans. Incidentally, mucin glycoproteins in the human gut, which could be substrates for ENGases, have been found to be capable of polymerizing as pH-tunable filaments. We hypothesized that these potential networked macromolecular assemblies could form and function akin to cellulosomes. Moreover, the structural insights of these protein oligomers can inspire approaches for the rational design of novel nanobiomaterials for biotechnological and/or therapeutic applications.

(P165) Serum glycomic profile as a predictive biomarker of recurrence in patients with differentiated thyroid cancer

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Recurrent thyroid cancer following curative intent thyroidectomy accounts for rising mortality rates, but current surveillance strategies fail to adequately identify these patients. Serum-based glycomics offers a unique opportunity to systematically monitor tumor-associated molecules and evaluate their value as tumor specific targets for tumor surveillance. Prior studies indicate that glycosylation is altered in thyroid cancer, but the role of glycosylation in thyroid cancer recurrence is unexplored. We performed serum N-glycomics of patients with recurrent thyroid cancer following thyroidectomy (N=13) versus non-cancer controls (N=15) and assessed global glycan features as well as individual glycans. We discovered that reduced galactosylation of biantennary N-glycans predicts thyroid cancer recurrence with an AUC of 82%. This novel non-invasive biomarker for recurrent thyroid cancer could have a major impact on the care of thyroid cancer patients, highlighting the potential role for serum glycomics in identifying disease phenotypes for personalized medicine.

(P166) Mapping the effects of glycosylation on the structure and function of IgG variable domains

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Asparagine-linked (*N*-linked) glycosylation is a common post-translational modification that can augment the structural and functional properties of immunoglobulin G (IgG) antibodies in a manner dependent on *N*-glycan location and/or composition. While conserved Fc *N*-glycosylation is well defined, far less is known about the *N*-glycans that are present in ~15-25% of IgG variable domains. Variable region *N*-glycosylation has not yet been systematically studied owing to the paucity of available strategies for selectively manipulating *N*-glycosylation and cataloging the resultant effects. Therefore, this work applies a high-throughput *Escherichia coli*-based glycomutagenesis strategy to interrogate the consequences of both *N*-glycan location and composition on the structure and function of IgG variable domains.

(P167) Comparing nematode glycomes – what's still to be learnt?

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The past two decades have seen immense progress in terms of our knowledge and understanding of the glycosylation capacities of a wide range of organisms. In our own studies, we have focused on the N-glycomes of 'lower' eukaryotes. Not only have the techniques and deepness of the data improved, but we can really say that simple organisms do not have simple glycomes. This is certainly true of the model nematode *Caenorhabditis elegans* and its parasitic relatives. An incredible selection of modifications of the mannosylchitobiosyl core, many seemingly unique to *C. elegans*, include not just a-fucosylation, but also various forms of a- and b-galactosylation and methylation. In many other nematode species, though, it is the antennae which are the major source of variability, whereby LacdiNAc or chito-oligomer chains often with fucose and/or phosphorylcholine residues have been found by a number of research groups in parasitic helminths. Only now, though, have we proven some examples of these in *C. elegans*, thus raising the prospect that the model worm has a not dissimilar glycogenomic capacity in terms of antennal N-glycan modifications as, for instance, *Oesophagostomum dentatum* or *Dirofilaria immitis*. However, despite over twenty years of glycomic research, we still lack knowledge as to many of the glycosidic linkages or their enzymatic origin. Other than the obvious orthologues of mammalian glycosyltransferases, clues as to which enzymes are involved are few, but mining the genome of *C. elegans* will hopefully result in a greater understanding of how it synthesises its seemingly ever more complicated glycome. Furthermore, glycan array studies are valuable for identifying nematode epitopes interacting with the mammalian immune system.

(P168) Tackling a tripartite glycan conundrum: Flexibility/Sparse structural data/Signal resolution

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Despite the vast molecular diversity of glycans, we face common, and often interconnected, challenges in their NMR structural studies, namely: 1) discrimination of distinct conformations amongst a conformation-rich landscape, 2) limited structural data for more reliable molecular modeling and 3) spectral overlap, especially observed for homopolymers. We have attempted to address these issues by improving sensitivity and spectral resolution, though both cannot be simultaneously achieved. We will show that labile ^{1}H signals can help alleviate two of these challenges increase the repertoire of structural data available and provide a path to discriminate potentially "biologically active" conformations and to improve the quality of both, the acquired data and the derived structural models. We will also present our ongoing efforts to enhance the spectral resolution to enable the structural studies of larger homo- and hetero-oligosaccharides.

(P169) Distinguishing patients with benign breast lesions and breast cancer by serum N-glycan analysis

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Early detection of breast cancer drastically increases the survival of breast cancer patients and provides multiple treatment options. While mammography is the standard tool for breast cancer screening, it often results in unnecessary biopsies of benign lesions. Incorporating additional assays could improve the performance of breast cancer screening. Here, we investigate the serum N-glycan profiles of patients with benign breast lesions or breast cancer (either DCIS or invasive) and identify N-glycans and N-glycan classes that can classify these patient groups. Serum samples from patients with benign lesions (n=199), DCIS (n=30), or invasive ductal carcinoma (n=69) were used. An amine-reactive slide was used to immobilize serum glycoproteins from 1 microliter of samples spotted on to the slide. The samples were delipidated and desalted with washes of Carnoy's solution and water. Peptide N-glycosidase F (PNGase F Prime) was sprayed on the samples to cleave the N-glycans, and a layer of matrix was applied. The released N-glycans were detected across the samples by MALDI-QTOF imaging mass spectrometry. Significant differences in N-glycan and N-glycan class intensities were determined by linear regression modelling, and the performance of N-glycans to classify disease status was evaluated using the area under the receiver operating characteristic curve (AUROC). When comparing the benign samples to the DCIS samples, a bisecting digalactosylated monosialylated N-glycan (2179.738 m/z) and a fucosylated biantennary digalactosylated disialylated N-glycan (2413.812 m/z) had significant differences in intensities. Additionally, 24 N-glycans associated with age and 12 N-glycans associated with BMI. The samples were stratified by patient age (younger than 40, 40-49, 50-74, older than 74) and BMI (less than 18.5,18.5-24.9, 25 to 29.9, more than 29.9) to identify

cancer-associated N-glycan intensity alterations in specific subsets of patients. The age ranges reflect the current United States Preventative Services Task Force mammogram recommendations, with only women aged 50-74 being recommended for biennial screening. The BMI ranges are the established classifications for underweight, healthy weight, overweight, and obese. In healthy weight women aged 50-74, two fucosylated bisecting monogalactosylated N-glycans with or without a sialic acid (2163.743 m/z and 1850.666 m/z, respectively) had significantly lower intensities in the cancer samples (n=11) and benign samples (n=45). Using these two N-glycans, age, and BMI, a AUROC of 0.899 was calculated with an optimal sensitivity of 82% and specificity of 84%. These findings indicate that serum N-glycan profiling is a promising approach for providing clarity to abnormal mammogram interpretations in the healthy weight women currently recommended for mammograms.

(P170) Application of human oral lectin ZG16B as a microbial glycan analysis probe (mGAP) to decode host-microbe interactions

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The oral cavity, one of the primary interfaces for host-microbe interactions, features complex microbiota. The interaction between the oral microbiome with the host proteins often involves the recognition of microbial cell wall carbohydrates by the carbohydrate-binding proteins residing in the oral cavity, lectins being one of them. Zymogen granule protein 16 homolog B (ZG16B) is one such less explored human soluble lectins, expressed in high abundance in the serous and seromucous acinar cells of the submandibular and sublingual glands in the oral cavity. To elucidate the function of ZG16B, we generated a microbial glycan analysis probe deploying recombinant lectin conjugated to fluorophore or biotin. Cy5 conjugated ZG16B was shown to bind to the cell wall peptidoglycan of oral commensal *Streptococcus vestibularis* isolated from dental plaques. ZG16B showed a bacteriostatic effect on *Streptococcus vestibularis* by aggregating the microbes, but not on other non-binding oral Streptococci, e.g. *Streptococcus oralis*. We further demonstrated that ZG16B binds to salivary mucin MUC7, but not to other predominant salivary mucins, e.g. MUC5B. ZG16B forms a ternary complex *in vitro* by recruiting MUC7 on *S. vestibularis*, enhancing clustering among the microbes. Our data suggest that ZG16B possibly regulates the overgrowth of oral commensals on the tooth surface by aggregation, followed by mucus-assisted clearance. The function of ZG16B reveals one of the possible mechanisms for maintaining bacterial homeostasis in the oral cavity via lectin-mediated host-microbe interaction.

(P171) SPR analysis on the interactions between heparin and SARS-CoV-2 spike protein RBDs from different variants

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The COVID-19 pandemic has become a major human health concern. The pathogen responsible for COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), invades its host through the interaction of its spike (S) protein with a host cell receptor, angiotensin-converting enzyme 2 (ACE2). In addition to ACE2, heparan sulfate (HS) on the surface of host cells also plays a significant role as a co-receptor. In this study, SARS-CoV-2 SP receptor binding domain (RBD) wild type (WT), Delta and Omicron variants were expressed in Expi293F cells and used in the kinetic and structural analysis on their interactions with heparin by Surface plasminogen resonance (SPR). Detailed kinetic and structural analysis of the interactions of SARS-CoV-2 SP RBDs with heparin provides important information for designing anti-SARS-CoV-2 molecules.

(P172) Epitope-focused immunogen design: Glycan masking of H7 influenza virus HA head domain

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Influenza virus rapidly mutates and escapes the immune system response. Antibodies targeting the viral surface glycoproteins are unable to recognize the antigen if mutations occurs at the binding interface. In particular, the creation of a glycosylation

site and the addition of a sugar chain at the antibody interface can completely abrogate antibody binding. This phenomenon, referred as glycan masking, has been adopted in epitope-focused vaccine design to hide specific epitopes associated to lower therapeutic effects and to better expose sites of vulnerability to the immune system.

In this work, we applied the glycan masking technique to design immunogen candidates based on the H7 hemagglutinin head (H7-head) glycoprotein of influenza virus, with a particular focus on the Trimer Interface site II (TI-II) epitope targeted by the protective human monoclonal antibody H7-200. We identified 25 antigenic sites on the H7-head, excluding the H7-200 epitope, which are highly prone to glycosylation. Each site is well exposed on the surface of the protein and contains one of the three residues forming the sequon for N-glycosylation NxS/T, in which the residue x in second position can be anything except for a proline.

We then combined different glycosylation sites in 20 H7-head variants: ten immunogen candidates presented three extra glycosylation sites in addition to the native one present on the H7-head (Tri mutants), seven presented five extra glycosylation sites (Penta mutants) and three presented seven extra glycosylation sites (Hepta mutants). The Rosetta Suite, a program for protein modeling and design, was used to: 1) insert the sequon for N-glycosylation in the structure of H7-head; 2) model the 3-dimensional conformation of the glycan chains; 3) dock a panel of known antibodies at their respective binding site, and 4) predict changes in antibody binding for each antigen-antibody pair.

Of the 20 candidates, only ten expressed well as recombinant proteins in HEK-293 cells, suggesting that certain glycosylations might lead to disruption in the folding of the protein, and thus might not appear during viral evolution.

Among the remaining 10 variants, each bound to mAb H7-200, as predicted. Many H7-head variants partially or completely lost binding of mAb H7-167, an antibody targeting the receptor binding site (RBS) on the HA head domain, and one variant H7-head construct lost binding of both H7-167 and H7.5, an antibody targeting the apex domain on HA head. Binding to the TI-I epitope, retained by all the ten H7-head variants, was tested with the broadly protective antibody FluA-20, which recognizes most influenza type A subtype viruses (subtypes H1 to H12 and H14-H15). The H7-head variants will be tested *in vivo* in future as candidate vaccine immunogens to specifically elicit an immune response focused on the more conserved TI epitopes of H7-head domain.

(P173) Wide occurrence of O-glycosylation Implicate Diverse Roles in the Extended Granin-Family

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Protein glycosylation is a major PTM found on important classes of proteins both soluble and embedded in the plasma membrane and glycocalyx. Protein glycosylation is undoubtedly important for the single cell and the complex organism and when glycosylation is impaired disease occurs. Depending on how affected the glycosylation apparatus is, milder diseases may occur, but when protein glycosylation is more broadly impaired severe diseases develop, most with clear metabolic and neurological involvement, and total loss of protein glycosylation is incompatible with life. Progress in discerning aetiology and approaching molecular mechanisms involving glycoproteins in neuronal development and metabolic signalling has been halted by technical limitations in studying protein glycosylation. Now we specifically mapped O-glycosylation sites in neuronal/neuroendocrine glycoproteins by performing targeted glycoproteomics on multiple neuronal and endocrine tissues and cell lines. Surveying the resulting >7.000 O-GalNAc-type glycosites identified in >3.000 proteins, we identified multiple glycosylation sites within major protein classes involved in biological niches such as perineural nets, synapse formation, endocytosis, cell-cell communication and granulogenesis. Thus, we hypothesize that O-linked glycans may impact such processes by i) directly stabilizing protein segments thus changing local conformations ii) providing the glycoprotein with mass and charge to engage in far-reaching molecular interactions and iii) increasing stability and changing the potential to engage with the plasma membrane. Interestingly, our map revealed abundant glycosylation in all members of the large secretogranin family that is essential for condensation and release of neuropeptide and biogenic amine signaling molecules in both neuronal and endocrine systems. To explore the nature and function of this secretogranin glycosylation we have taken a genetic engineering approach where selective ablation of glycosyltransferase genes enables removal of single types of protein glycosylation, while keeping the remainder of the glycome intact. We have generated a panel of cell lines deficient in O-GalNAc- and GAG-type glycosylation pathways associated with secretogranin glycosylation. Collectively our data show that the secretogranins contain high, but subsaturated, stoichiometry of O-glycans and that this glycosylation is important for proper multimerization of the key member of the secretogranin-family: Chromogranin A. In line with these results, glyco-KO cells have higher capacity for the neurotransmitter noradrenaline in concert with slightly expanded size of neurotransmitter/hormone containing dense core vesicles. In summary, we have generated a map of the neuro/endocrine glycosylation landscape, and explored their role as modulators of neurotransmitters specifically in the secretogranin-family using genetic engineered cell lines.

(P174) Canine Glycoproteomics and the Search for Biomarkers of Filarial Disease

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Parasitic diseases caused by filarial nematodes place an incredible burden on those who are infected and the societies in which they are endemic, but much remains unknown about disease progression and interaction with the immune system. Early detection of these infections prior to the establishment of patent infection is paramount in disease control. *Dirofilaria immitis*, the causative agent of heartworm disease in canines, is important both as a veterinary disease throughout the world and as a potential model for other filarial nematodes. Although modern preventatives are effective, resistance to the treatments is on the rise and the changing climate has had impacts on the range for the parasite. In our study we focus on both proteomics and glycoproteomics, with the application of the lectin Fbs1 to enrich glycopeptides in our samples to increase the depth of our analysis. Through the application of proteomics, we seek to understand both the host serum response to infection and the secretion of bioactive compounds from the parasite. Each of these may act as biomarkers, enabling earlier detection of infections and could act as potential targets for novel therapies.

(P175) Roles of Matriglycan and the Underlying M3 Glycan on Alpha-Dystroglycan in Congenital Muscular Dystrophy

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α-Dystroglycan (α-DG) is uniquely modified on *O*-mannose sites by an M3 extended glycan terminating in the repeating disaccharide (-Xylα1,3-GlcAβ1,3-)$_n$ termed matriglycan, which is a receptor for laminin-G domain-containing proteins and employed by old-world arenaviruses for infection. Deletion of post-ribitol processing glycosyltransferases, eliminates the ability of α-DG to bind the IIH6 antibody, Laminin, or Lassa virus GP1. Using chemoenzymatically synthesized matriglycans printed as a microarray, we demonstrate length-dependent binding to Laminin, Lassa virus GP1, and the clinically-important antibody IIH6. Utilizing an enzymatic engineering approach, the *N*-linked glycoprotein fetuin was converted into a IIH6-positive Laminin-binding glycoprotein. Engineering of the surface of cells deficient for either α-DG or *O*-mannosylation with matriglycans of sufficient length recovers infection with a Lassa-pseudovirus. Further, free matriglycan in a dose and length dependent manner inhibits viral infection of wildtype cells. These results indicate that matriglycan alone is necessary and sufficient for IIH6 staining, Laminin and LASV GP1 binding, and Lassa-pseudovirus infection and support a model in which it is a tunable receptor for which increasing chain length enhances ligand-binding capacity. Finally, we also present data that the underlying M3 glycosyltransferases show high selectivity of substrates that have likely evolved to ensure that matriglycan is only added to 2-3 threonine residues on α-DG in the mammalian proteome.

(P176) Glycocode of the tumor microenvironment as a novel immune check-point

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Alterations in glycosylation is a hallmark in pathogen recognition by the host which mediates cellular communication during inflammation but also the resolution-phase of inflammation. Homeostatic and inflammatory processes affect the glycan signature of glycoproteins expressed on the cell surface, or secretory proteins, due to up- and down-regulation of glycosylation genes. We study those glycan signatures that are recognized by glycan binding receptors such as C-type lectins and Siglecs expressed on diverse set of myeloid and lymphoid immune cells, that modify intracellular signalling and immunological outcome. In particular the microenvironmental glycosylation signatures (the glycocode) that alter during inflammation and cancer are studied that drive immune responses towards immunity or tolerance and open new venues for immune interference.

We identified new mechanisms of immune tolerance through the modification of glycosylation of tumours (melanoma, pancreatic cancer and glioblastoma). In particular high sialylation of tumours results in the increase of FoxP3 CD4+ T cells (Treg), differentiation of monocytes into TAMs and lower frequencies of effector T cells (Teff) and NK cells at the tumour site. In contrast, low sialylation of tumours converts the frequencies Treg/Teff to favourable anti-tumour immunity. These sialic acids can be used for the active induction of antigen specific immune tolerance by DC when coupled to a specific antigens, such as OVA, or peptides of MOG or derp-1 thereby setting the resolution phase and repairing auto-immunity such as multiple sclerosis or allergies.

Post-translational processes such as glycosylation, uncover new communication between tumor and immune cells. Because these glycosylations can be immune stimulatory or inhibitory we implement our discoveries in the treatment of cancer and auto-immune diseases.

(P177) Keratan sulfate biosynthesis occurs via O-linked mannose (O-Man) on KIAA1549

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Keratan sulfate (KS) is a glycosaminoglycan with unique functional roles in cornea, brain and cartilaginous tissues. KS is based on poly-N-acetyllactosamine (poly-LacNAc) with varying 6-sulfation pattern that extend from N-glycans (KS-I), O-GalNAc glycans (KS-II) or O-linked mannose (O-Man) glycans (KS-III). KS-I and KS-II modified proteins have been characterized, however, protein carriers and biosynthetic pathways of KS-III have remained elusive for decades.

We previously identified the transmembrane KIAA1549 protein as a POMT1/2 substrate with >60 O-Man glycan sites, and hypothesized that KIAA1549 may carry KS-III modifications based on its extensive O-Man glycosylation pattern, its predominant expression in retina/brain and the genetic defects which link *KIAA1549* to ocular phenotypes and pediatric brain cancer (pilocytic astrocytoma).

To test this hypothesis, we first established a CRISPR/Cas9 glycoengineered CHO cell panel. CHO^{WT} cells lack capacity for KS biosynthesis and we knocked-in selected *N*-Acetylglucosaminyltransferases and sulfotransferases to enable KS biosynthesis (CHO^{KS}) before knock-out of *POMT1* ($CHO^{KS/POMT1\ KO}$). The predicted extracellular domain of KIAA1549 ($KIAA^{62\text{-}950}$) was stably expressed for affinity purification from ell media and characterization by mass spectrometry.

We observed O-Man glycosylation of $KIAA^{62\text{-}950}$ expressed in CHO^{WT}, including core M1-type (Neu5Ac-Gal-GlcNAc-Man-O-Ser/Thr) structures, and demonstrate that extensive O-glycan modifications are added to $KIAA^{62\text{-}950}$ only in CHO^{KS} cells. Using the KS-specific antibody MZ15 and PNGase F digestion, we confirm that KS-biosynthesis occurs via O-glycans in CHO^{KS} and that $KIAA^{62\text{-}950}$ lacks KS glycosylation when expressed in $CHO^{KS/POMT1\ KO}$ cells. Finally, we demonstrate poly-LacNAc extension of O-Man glycans (KS-III) on $KIAA^{62\text{-}950}$ expressed in CHO^{KS} by mass spectrometry.

Our continued efforts focus on genetic dissection and analysis of linkage positions for the KS-III modification, and we are currently exploring KS-III biosynthesis in relation to *POMGNT1*, *POMGNT2* and *MGAT5B* KO in CHO and HEK293 cells, with ambitions to unravel the molecular details and enzymatic pathways that control KS-III biosynthesis in mammalian cells.

(P178) Advances in Native Mass Spectrometry-based Glycan Library Screening

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The associations of glycans and glycan-binding proteins (GBPs) mediate many important physiological and pathophysiological processes. Identifying these interactions is essential to understanding the diverse biological roles glycans play and the development of new disease treatments and diagnostics. Knowledge of the repertoire of glycans recognized by most GBPs and their affinities is incomplete. Mass spectrometry-based shotgun glycomics (MS-SG), which combines native MS (nMS) screening methods and libraries of glycans extracted from natural sources (e.g. cells, tissue or biofluids), is a promising approach for mapping glycan interactions with GBPs. However, nMS screening of glycans at unknown concentrations doesn't allow for affinities and, correspondingly, specificities to be measured. Here, recent advances in quantitative MS-SG methodologies are described. The first approach combines nMS screening data with relative glycan concentrations measured for a fluorophore-labeled library by liquid chromatography to produce affinity rankings. This method was tested using a series of sialic acid-binding lectins and natural *N*-glycan libraries. While easily implementable, the affinity rankings obtained with this approach are sensitive to the nature of the fluorophore added. Recognizing the need to eliminate the undesirable effects of glycan labeling, concentration-independent (COIN)-nMS, which exploits the newly developed slow mixing mode (SLOMO) nMS technique, was conceived. This label-free approach, which can be applied to mixtures of glycans at unknown concentration, enables the affinities of all detected interactions to be measured simultaneously. The screening results obtained for human, bacterial and viral GBPs with natural libraries of glycans, glycopeptides and glycolipids illustrate the power of COIN-nMS for quantitative MS-SG applications.

(P179) Multilayered regulation of p38 signaling by O-GlcNAcylation in cardiac myocytes

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Aberrant activation of the stress kinase p38 promotes cardiac pathology and p38 inhibition is tested as a potential therapeutic approach to treat heart failure. While p38 inhibitors effectively improve cardiac pathology in animal models, their outcomes in clinical trials of cardiac disease are less successful. This could be due to 'escape' mechanisms, that enable p38 to overcome

long-term inhibition. p38 is phosphorylated and activated by canonical upstream kinases MKK3/6 and by poorly understood non-canonical activation mechanisms. A better understanding of the mechanisms that govern p38 phosphorylation and activation can therefore improve the efficacy of p38 inhibitors in clinical practice. O-GlcNAcylation is a regulated and reversible protein modification that in terms of dynamics and breadth of target proteins resembles phosphorylation. O-GlcNAcylation is regulated by cycling enzymes O-GlcNAc Transferase (OGT) and O-GlcNAcase (OGA) that catalyze the opposing reactions of O-GlcNAc addition and removal. Previous work demonstrated that OGT and p38 physically interact and found that p38 is a substrate of OGT for O-GlcNAcylation. Using cultured primary neonatal cardiomyocytes as an experimental system, we examined whether altering O-GlcNAcylation impacts p38 phosphorylation during basal and hypertrophic signaling. Treating myocytes with OSMI-1, a chemical inhibitor of OGT, significantly increased the baseline phosphorylation of p38 (3.9-fold increase $\pm$ 0.46, $P<0.001$ vs. vehicle) and impaired the physiologic growth response of cardiomyocytes. Mechanistically, we found that knockdown of the canonical upstream kinases MKK3/6 prevented OSMI-1-induced phosphorylation of p38 and the phosphorylation of the downstream target Hsp27. Additionally, knockdown of Tab1, a scaffold protein that promotes p38's non-canonical activation, prevented OSMI-1-induced phosphorylation of p38. Using metabolic labeling with clickable unnatural sugar analogs we found evidence that a subset of p38 is potentially O-GlcNAcylated in cardiomyocytes. The same conclusions were also drawn for the scaffold protein Tab1. Taken together, the data indicate that O-GlcNAcylation is a key regulatory modality that significantly impacts p38 signaling in cardiomyocytes through effects on both canonical and non-canonical pathways. Further investigation into the functional relationship linking O-GlcNAcylation, p38 and p38-upstream regulators can ultimately lead to improved approaches for targeting p38 in cardiac disease.

(P180) A tissue-specific view on sugar metabolism

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Congenital Disorders of Glycosylation (CDG) form a fast growing group of genetic defects, that are characterized by abnormal glycosylation of proteins and lipids. Already more than 150 different genetic diseases have been discovered in a variety of glycosylation pathways, such as O-mannosylation, glycosaminoglycan synthesis, lipid glycosylation, and N-glycosylation.

Identification of genetic defects has spurred research into the mechanisms of glycosylation, for example by revealing novel glycan structures and unexplained clinical symptoms. The main interest of our group is to define novel biological mechanisms in sugar metabolism, i.e. the synthesis of nucleotide sugars, the building blocks of glycans. Based on the classical glycobiological view, humans have a generic set of individual sugar supply pathways in all cells that generate nucleotide sugars such as CMP-sialic acid and GDP-mannose for glycosylation. However, patients with different genetic defects in a single sugar supply pathway, for example for GDP-mannose or CMP-sialic acid, present with completely different and contrasting tissue-specific clinical symptoms, which is not explained by our current knowledge. For example, genetic defects in sialic acid metabolism can present with severe neurological deterioration or adult onset muscular atrophy. We established mass spectrometry methods for analysis of nucleotide sugars and sugar phosphate intermediates, both at steady-state and dynamically by stable isotope labeling. Thereby, we aim to unravel novel mechanisms in sugar metabolism, such as our previous identification of the biosynthesis of CDP-ribitol, the presence of redundant enzymes in the dolichol-P-mannose and sialic acid pathways.

What we start to see is that sugar metabolism is to be regarded as a network of reactions, rather than a set of individual sugar supply pathways. Moreover, this network of reactions depends on the conditions and tissue under study, which we aim to investigate in patient-derived induced pluripotent stem-cell models of heart, muscle and brain. The resulting organ-on-chip models allow parallel analysis of physiology and metabolism. This presentation will cover the background into the topic, the conceptual framework and our ongoing work to uncover tissue-specific mechanisms in sugar metabolism.

(P181) Glycoengineering a more effective CAR-T cell therapy

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Introduction and Objective: Cancer is the second leading cause of death in the US, and there is great emphasis in finding safe and effective therapies. One of these therapies is adoptive chimeric antigen receptor (CAR)-T cell therapy, consists of a patient's own immune cells (T cells) that are harvested, expanded and engineered to target cancer. However, CAR-T cell therapy requires therapeutic improvements, such as boosting efficiency, efficacy and improving persistence *in vivo*. T cells express surface carbohydrate chains (or glycans) capable of binding glycan-binding proteins (lectins) compromising their survival and effector

function. There are compelling experimental data that ß-galactoside-binding, galectins, which are ubiquitously expressed in host tissues and elevated in patients with B cell lymphomas, can bind T cell surfaces to suppress T cell immunologic anti-tumor activity and/or shorten T cell viability. We hypothesize that galectins play a critical role in the efficacy of CAR-T cell therapy and cell surface glycan modification of CAR-T cells to evade galectin-binding capabilities can increase persistence and functional activity in vivo.

Methods: We analyzed the surface glycome and galectin-binding activity of control naïve human T cells, *ex vivo*-expanded human T cells as well as CAR-T cells. We explored binding ability of common immunosuppressive galectins, recombinant human galectin (rhGal)-1, rhGal-3, rhGal-9, by flow cytometry. Moreover, we analyzed the ability of rhGal-1 to elicit a pro-apoptotic activity in cell death assays and examined the expression of glycosyltransferases, *ST6Gal1*, *GCNT1*, *GCNT2* and *MGAT5*, involved in the biosynthesis and the inhibition of galectin-binding glycans using RT-qPCR. All methods were conducted a minimum of 3-times and tested for statistical significance using Student's paired *t*-test :$*p<0.05$, $**p < 0.001$.

Results: Compared with binding to naïve T cells, we found that *ex vivo*-expanded human T cells and CAR-T cells exhibited robust binding to Gal-1 and Gal-9 and corresponded with elevated susceptibility to Gal-1-dependent pro-apoptotic activity ($p < 0.05$). Alpha 2,6 sialyltransferase, ST6Gal1, gene expression was suppressed in CAR-T cells that was inversely related with Gal-1-binding in temporal flow cytometry assessments. Lastly, compared with CAR-T cell controls, Gal-1-binding in ST6Gal1 overexpressing CAR-T cells was reduced, suggesting that ST6Gal1 expression and α2,6 sialylation to surface glycans can potentially enhance longevity of CAR-T cells.

Conclusions-Implications: The data suggest that understanding a human T cell's surface glycome can provide evidence of the immunosuppressive potential that can compromise the persistence and/or functional activity of CAR-T cells. These findings also indicate that glycoengineering of CAR-T cells with ectopic ST6Gal1 expression or other α2,6 sialylation methods can help antagonize Gal-1-binding and build a more durable, immunoprotected CAR-T cell.

(P182) Functions of LDLR-related protein 1 (LRP1) O-glycosylation

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Endocytosis, the process by which cells specifically take up macromolecules, is fundamental to all cells. In this way cells control the protein composition on the cell surface and in extracellular fluids. The low-density lipoprotein receptor (LDLR) and LDLR-related proteins (LRPs) comprise a family of structurally related endocytic receptors with essential functions in protein reabsorption from urine, cholesterol clearance and endocytic trafficking of neuronal and cerebrospinal fluid (CSF) proteins at the blood-CSF and blood-brain-barrier. While LDLR and VLDLR have highly restricted ligand selectivity, the large LRP1 and LRP2/Megalin receptors serve a plethora of functionally and structurally diverse ligands destined for degradation or transcytosis [1,2]. How the broad ligand specificities of LRP1 and -2 are accommodated and how different ligands can elicit different cellular responses is not completely understood and partly ascribed to their large complex structure with up to 4 ligand-binding domains comprised of clusters of extracellular LDLR class A complement type repeats (CR) [3,4]. Individual CRs are connected by short linkers where the sequence $\mathbf{C_6XXX\underline{T/S}C_1}$ is highly conserved. We discovered that $\mathbf{C_6XXX\underline{T/S}C_1}$ linkers carry a high-occupancy O-glycan [5] that is specifically regulated by GALNT11, a member of a family of up to 20 distinct GALNT isoenzymes that all initiate protein O-glycosylation [6,7]. We have now found that in mammalian cells 9 out of 9 consensus-sequence linkers present in LRP1 can be glycosylated and to explore the functional consequence of LRP1 linker glycosylation, we have established cell models with expression of full-length LRP1 with and without GALNT11 specific glycosylation. We have probed ligand binding and uptake specifically and globally in complex protein mixtures and obtained data that suggests that in addition to the well-defined structural requirements in the CR repeats, linker O-glycans may provide the mass and charge to direct differential ligand interaction and LRP1 receptor function.

(P183) Activity-dependent Golgi satellite formation in dendrites reshapes the neuronal surface glycoproteome

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Activity-driven changes in the neuronal surface glycoproteome occur with synapse formation, plasticity, and related diseases, but their mechanistic basis and significance are unclear. Here, we observed that N-glycans on surface glycoproteins of dendrites shift from immature to mature forms containing sialic acid in response to increased neuronal activation. In exploring the basis of these N-glycosylation alterations, we discovered that they result from the growth and proliferation of Golgi satellites scattered

throughout dendrites. Golgi satellites that formed with neuronal excitation were in close association with endoplasmic reticulum (ER) exit sites and early endosomes and contained glycosylation machinery without the Golgi structural protein, GM130. They functioned as distal glycosylation stations in dendrites, terminally modifying sugars either on newly synthesized glycoproteins passing through the secretory pathway or on surface glycoproteins taken up from the endocytic pathway. These activities led to major changes in the dendritic surface of excited neurons, impacting binding and uptake of lectins, as well as causing functional changes in neurotransmitter receptors such as nicotinic acetylcholine receptors. Neural activity thus boosts the activity of the dendrite's satellite micro-secretory system by redistributing Golgi enzymes involved in glycan modifications into peripheral Golgi satellites. This remodeling of the neuronal surface has potential significance for synaptic plasticity, addiction, and disease.

(P184) Composition and function of the blood-brain barrier glycocalyx in health and neuroinflammatory disease

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The blood-brain barrier (BBB) glycocalyx is the sugar rich structure lining the luminal surface of cerebral blood vessels that acts as the first barrier in the BBB. The BBB describes the combination of properties that tightly regulates blood vessel permeability and is exclusively found in the central nervous system vasculature. Despite its importance in the BBB, the glycocalyx is poorly understood; we do not fully understand its molecular composition or how it differs from the glycocalyx in other organs. However, its degradation has been shown to increase BBB permeability and neuroinflammation.

To better understand the structure of the BBB glycocalyx, we performed electron microscopy on the glycocalyx in brain, heart, and muscle tissues from healthy mice. We found that the BBB glycocalyx in the brain was thicker than the glycocalyx in the heart and muscle. These findings suggest that the thicker BBB glycocalyx may contribute to the tight regulation of molecules and cells between the blood and the brain. Furthermore, preliminary RNA sequencing and glycomics investigations have shown that the BBB glycocalyx may be enriched in specific glycans. In future studies, we will investigate the role of these glycans in the BBB glycocalyx, the barrier contribution of the glycocalyx to the BBB, and how its structure and composition change in neuroinflammatory disease.

(P185) Glycomimetic Antagonist Uproleselan (GMI-1271) Breaks E-selectin-mediated Chemoresistance in Acute Myelogenous Leukemia

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The concept of cancer progression being influenced by both intrinsic characteristics of the cancer cell as well as its interactions with the extrinsic tumor microenvironment has been known for over a century as proposed in Paget's "Seed and Soil" hypothesis with fundamental evidence of its existence provided by Isaiah Fidler. SialylLe^a and sialylLe^x expressed on tumor cells share a common trisaccharide domain that is the epitope for binding to E-selectin. E-selectin is an adhesion molecule expressed on endothelial cells lining the blood vessels and is extrinsic to the cancer cell in the tumor microenvironment. E-selectin is unique among the adhesion molecules in that its binding induces the NFk-B pathway of chemoresistance. Cancer cells arise from these niches post chemotherapy as evidenced by a fourfold ($p = 0.004$) increase in expression of sialylLe^x on AML cells from relapsed patients. Genomic screens of cancers have identified intrinsic mutations of cancer cells associated with poor prognosis. The mechanism of action in many cases, however, is extrinsic through E-selectin-mediated chemoresistance. An example is the FLT-III ITD mutation in AML which results in secretion of TNFa stimulating expression of E-selectin. Analysis of the data on FLT-III ITD AML patients from The Cancer Genome Atlas at the NCI reveals that poor survival indicative of FLT-III ITD AML patients only occurs when these patients also express high levels of the fucosyltransferase (FUT 7) required to synthesize sialylLe^x. Without high expression of FUT7, FLT-III ITD patients have identical survival curves to AML patients without this mutation, showing that although the mutation is intrinsic (seed) the mechanism is through extrinsic E-selectin-mediated chemoresistance (soil). To treat this widespread mechanism of cancer chemoresistance, a potent and specific glycomimetic antagonist (Uproleselan) was designed based on the bioactive conformation of sialylLe^x. AML cells are heavily glycosylated with sialylLe^x. In a Phase 1/2 clinical trial treating relapsed/refractory AML patients, the addition of Uproleselan to chemotherapy reversed predicted overall survival based on expression of sialylLe^x. That is, those patients with higher expression of sialylLe^x had significantly higher complete responses and significantly longer overall survival suggesting that Uproleselan was hitting its target in patients and inhibiting E-selectin-mediated chemoresistance. Given the supportive results from this trial, a Phase 3 randomized controlled trial is underway to verify the benefits of adding Uproleselan to salvage chemotherapy regimens in patients with relapsed/refractory disease. While genomics has identified mutations in cancer that correlate with poor prognosis, the mechanism of action is not

always understood. Here, we provide data that E-selectin plays a fundamental role in chemoresistance in different cancers and is susceptible to treatment with a potent and specific glycomimetic drug.

(P186) COG4 mutation in Saul-Wilson Syndrome selectively affects secretion of proteins involved in chondrogenesis in chondrocyte-like cells

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Saul-Wilson syndrome is a rare skeletal dysplasia caused by a heterozygous mutation in COG4 (p.G516R). Our previous study showed that this mutation affected glycosylation of proteoglycans and disturbed chondrocyte elongation and intercalation in zebrafish embryos expressing the COG4$^{p.G516R}$ variant. How this mutation causes chondrocyte deficiencies remain unsolved. CRISPR knock-in was used to generate the COG4$^{p.G516R}$ variant in the chondrosarcoma cell line SW1353 to study chondrocyte differentiation and protein secretion. COG4$^{p.G516R}$ cells display impaired protein trafficking and altered COG complex size, similar to SWS-derived fibroblasts. Both SW1353 and HEK293T cells carrying COG4$^{p.G516R}$ showed very modest, cell-type dependent changes in N-glycans. Using 3D cultures, we found that cells carrying COG4$^{p.G516R}$ variant made smaller spheroids and had increased apoptosis, indicating impaired *in vitro* chondrogenesis. Adding WT cells or their conditioned medium reduced cell death and increased spheroid sizes of COG4$^{p.G516R}$ mutant cells, suggesting a deficiency in secreted matrix components. Mass spectrometry-based secretome analysis showed selectively impaired protein secretion, including MMP13 and IGFBP7 which are involved in chondrogenesis and osteogenesis. We verified reduced expression of chondrogenic differentiation markers, MMP13 and COL10A1 and delayed response to BMP2 in COG4$^{p.G516R}$ mutant cells. Collectively, our results show that the SWS COG4$^{p.G516R}$ variant selectively affects the secretion of multiple proteins, especially in chondrocyte-like cells which could further cause pleiotropic defects including hampering long bone growth in SWS individuals.

(P187) Exo-enzymatic mapping of glycan mediated interactions on cell surfaces using diazirine-modified sugars

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Glycans expressed on the cell surface are involved in various macromolecular biological interactions. In general, these interactions have a low affinity which make them difficult to characterize using conventional biochemical techniques as most of the interactions are lost during purification steps. Metabolic feeding of photocrosslinking sugar analogs has enabled covalent capture of glycan-mediated interactions *in situ* however metabolic incorporation of sugar analogs is limited to certain cell types and also not efficient in all of them. In the present work we addressed the limitations of metabolic glycan engineering by labelling cell surface glycoconjugates with diazirine modified sugars exo-enzymatically. This method involves chemoenzymatic synthesis of diazirine-modified CMP-sialic acid (CMP-SiaDAz), and GDP-Fucose (GDP-FucDAz) followed by addition of SiaDAz and FucDAz to cell surfaces using respective bacterial glycosyltransferases. Cell surface SiaDAz-ylation can be performed in multiple cell types, and exogenous addition of sialyltransferase is not required to add SiaDAz to Daudi B cells. This method for extracellular addition of α2-6-linked SiaDAz enables UV-induced crosslinking of CD22, demonstrating the utility for covalent capture of glycan-mediated binding interactions.

(P188) Evaluating a Glycan Database And Glycopeptide Result Converter Using Human Serum Across Multiple Glycopeptide Search Engines

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Multiple glycoproteomics search engines are currently available, including Byonic, pGlyco3, Metamorpheus, just to name a few. Their utility is limited by a lack of harmonization with regard to input and output formats across these search engines. Thus,

glycopeptide search results from different programs cannot be directly compared, and users are relegated to coming up with ways to correlate resultant data. Moreover, various search engines apply different algorithms to calculate output "confidence scores". Here, we present a novel application to inter-convert/translate the outputs of several commonly used glycan database and search engine formats. We have applied our conversion program to human serum samples analyzed by different *N*-linked glycopeptide search engines, including pGlyco3, Byonic, and MetaMorpheus, and report confidence score dynamics for the results.

An *N*-linked glycan database for individual search engines was built for the Byonic, pGlyco3, and Metamorpheus formats. Identical parameter searches were run on these search engines to yield glycopeptide search results. The results from the different search engines were then converted into a common format using our glycopeptide database converter. Confidence score comparisons were subsequently compared by reverse tracing of the scan numbers for individual experiments. We used our glycan database converter on pGlyco3 glycan database input files for 154 human *N*-linked glycan compositions from the Byonic search engine's default *N*-glycan human plasma database. Glycopeptide identification was achieved using searches on pGlyco3, Byonic, MetaMorpheus search engines with the same searching parameters. Our glycopeptide result converter was applied to extract specific information including: MS2 scan number, peptide sequence, glycoform, retention time, and confidence score output from each search engine result. In the sample analyzed across five FAIMS collisional voltages (35V, 40V, 45V, 50V, 55V), pGlyco3 identified 3,038-6,801 glycopeptide-containing spectra (GCS), Byonic identified 4,660-9,737 GCS, and MetaMorpheus identified 3,140-6,818 GCS. Since our glycopeptide result converter retrieves MS2 scan numbers and confidence scores, conversions between confidence scores for different search engines could be analyzed based on the same MS2 scan. Without any confidence score filters, 30.1% (3,266 of 10,868 PSMs) of GCS reported a shared space at FAIMS CV 45V by the pGlyco3, Byonic, and MetaMorpheus search engines.

In conclusion, our glycopeptide converter enabled a direct comparison of the output results from pGlyco3, Byonic, and MetaMorpheus, while accounting for differences in the respective confidence scores.

(P189) Comparative site-specific N-glycoproteome analysis reveals aberrant N-glycosylation and gives new insights into mannose-6-phosphate pathway in cancer

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N-glycosylation is implicated in cancers and aberrant N-glycosylation is recognized as a hallmark of cancer. Here, we mapped and compared the site-specific N-glycoproteomes of colon cancer HCT116 cells and isogenic non-tumorigenic *DNMT1/3b* double knockout (DKO1) cells using Fbs1-GYR N-glycopeptide enrichment technology and trapped ion mobility spectrometry. Many significant changes in site-specific N-glycosylation were revealed, providing a molecular basis for further elucidation of the role of N-glycosylation in protein function. HCT116 cells display hypersialylation especially in cell surface membrane proteins. Both HCT116 and DKO1 show an abundance of paucimannose and 80% of paucimannose-rich proteins are annotated to reside in exosomes. The most striking N-glycosylation alteration was the degree of mannose-6-phosphate (M6P) modification. N-glycoproteomic analyses revealed that HCT116 displays hyper-M6P modification, which was orthogonally validated by M6P immunodetection. Significant observed differences in N-glycosylation patterns of the major M6P receptor, CI-MPR in HCT116 and DKO1 may contribute to the hyper-M6P phenotype of HCT116 cells. This comparative site-specific N-glycoproteome analysis provides a pool of potential N-glycosylation-related cancer biomarkers, but also gives new insights into the M6P pathway in cancer.

(P190) Mass Spectrometry Characterization of Gangliosides from Mouse Optic Nerve Samples

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Gangliosides (GS) are a composed of hydrophilic glycan and hydrophobic ceramide and comprise a family of glycosphingolipids which vary in the distribution of sialic acid residues[1]. Due to the nature of their structure, GS predominantly colocalize in cells, having the hydrophilic glycan portion of this structure interacting with extracellular environment and the hydrophobic ceramide moiety embedded in the outer leaflet of the plasma membrane[2]. Their chemical composition and expression level is cell specific and their alteration in the nervous system correlate with developmental stages, as well as disease[3]. Traditional techniques to detect GS composition include thin layer chromatography (TLC) followed by immunoreactivity assays or chemical reactions with the sialic acid portions. Combinations of these techniques yield the oligosaccharide moieties but are less robust at determining the ceramide portion. The glycans separated from the lipid portion can also be detected with high precision by using

Nuclear magnetic resonance (NMR). Here, we describe a method that allows for the identification and relative quantification of gangliosides through liquid chromatography-mass spectrometry (LC MS-MS). The methodology described includes extraction, MS settings, identification steps, and suggested statistical analysis. The identification of gangliosides is achieved using mouse optic nerve and allows for the characterization of each sample's lipidome. Two deuterium labeled ganglioside and a general lipid class standards are utilized for quantification and extraction efficiency. The samples are analyzed with reversed-phase high-performance liquid chromatography (HPLC) coupled to a Q Exactive mass spectrometer.

(P191) Deciphering the functional implication of Gardnerella sialidase activity in host interactions using isogenic bacterial strains

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The normally lactobacilli-rich human vaginal microbiome can become overgrown with diverse fastidious and anaerobic bacteria, a clinical condition known as bacterial vaginosis (or BV). It is often associated with adverse reproductive and sexual health outcomes. A key diagnostic feature of BV is the abundance (relative to lactobacilli-dominant, no-BV) of sialidase activity in vaginal fluids. This activity appears to be of bacterial origin. Women with BV frequently harbor known sialidase-producing bacterial genera like *Gardnerella, Prevotella, Bacteroides*. Abundance of *Gardnerella* is prevalent in BV and the bacteria also confer BV-like phenotypes upon vaginal infection in mice. Sialidases have been reported to correlate with a number of clinical and microbiological aspects of BV. However, the inability to genetically manipulate *Gardnerella* and other BV bacteria have limited our causal understanding of the role(s) of sialidase in vaginal host-microbiome interactions. Here we characterized strains of *Gardnerella* from the JCP8070 background that spontaneously differ in the lengths of polycytosine tracts within the *nanH3*-encoded sialidase. Phenotypic analysis of individual daughter colonies from five isogenic parental strains with alternate tract lengths showed dramatically different levels of sialidase activity amongst colonies arising from one strain compared to all others. Regardless of the polyC length, JCP8070 strains were able to use glucose, and free sialic acid equally well as primary carbohydrate sources to support growth *in vitro*. However, after a prolonged lag phase, the out-of-frame strain (nonfunctional NanH3) also grew on bound sialic acid (sialyllactose) and showed a corresponding increase in levels of sialidase activity. In the mouse vagina, the in-frame *nanH3* strain conferred higher vaginal sialidase activity than the out-of-frame strain. Vaginal sialidase levels increased in mice receiving the out-of-frame strain from 6 to 24 hours, following exogenous sialyllactose administration. However, by 24 hours, similar levels of sialidase were observed between experimental groups infected with in-frame versus out-of-frame *nanH3* strains. Our current working model is that greater access to free (or liberated from sialoglycans) sialic acid provides a competitive advantage for even small numbers of cells with in-frame *nanH3* to expand, to a point. We hypothesize that once sialidase activity levels of the culture reach a threshold, it is sufficient to gather this resource on behalf of the population. We are currently exploring additional aspects of *Gardnerella* behavior and pathogenesis using this unique set of isogenic strains bearing different nanH3 polyC tract lengths.

(P192) Circulating glycopeptide markers differentiate between early- and late-stage epithelial ovarian cancer

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Epithelial ovarian cancer (EOC) is a disease that continues to have dismal survival rates, in the face of generally late diagnosis and limited treatment options. InterVenn Biosciences' GLORI™ assay is the world's first serum-based glycoproteomic, CLIA-CAP compliant laboratory-developed test that differentiates between benign and malignant adnexal masses. While analyzing clinical data from the studies used to develop GLORI, we observed a distinct signature of circulating N-glycoproteins that differentiated late-stage (stage III/IV) and early-stage (stage I/II) EOC. Qiagen's Ingenuity pathway analysis of these data predicted that these biomarkers are localized downstream of cytokine signaling. Notably, the biomarkers of interest appear to indicate the presence of the sialyl Lewis X (sLe^x) epitope on N-glycans of liver-derived circulating glycoproteins. Previous studies have consistently shown an association of this epitope with metastatic cancer in general. In addition, we applied machine learning tools to the identification of circulating glycoproteomic biomarkers that would differentiate between early and late stage

EOC. The data generated - if validated in prospective clinical studies - may help in developing a blood-based test for staging, triaging, or treatment recommendations as well as for monitoring recurrence and metastatic transformation of EOC. Lastly, our data suggest that sLe^x might be an attractive target for the treatment of metastatic EOC, or the prevention of progression of earlier stage EOC to metastatic disease.

(P193) Convergent Chemoenzymatic Synthesis of O-GalNAc Glycan Rare Core 5, 7, 8 and Their Sialylated Forms

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The three O-GalNAc glycan (also known as mucin glycan) rare cores with α-linked extension (cores 5, 7, 8) were prepared from a universal precursor in a convergent manner, yielding exclusively α-configuration. Efficient synthesis of their α2-6sialylated forms was achieved by using Photobacterium damsela α2-6sialyltransferase. These structures together with β-extended cores 1−4, 6, and their sialylated forms were then fabricated into a unique O-GalNAc core microarray to profile the binding of several clinically important GalNAc-specific lectins. Among all O-GalNAc cores, it is revealed that only Tn, (sialyl-)core 5, and core 7 are binders of WFL, VVL, and SBA, while DBA only recognized core 5 and sialylated forms. Jacalin is the only lectin bound to core 8 (but not core 5), and to other (sialyl-)cores without C6-modification of the core GalNAc. Furthermore, activity assays of human α-N-acetylgalactosaminide α2-6sialyltransferases (ST6GalNAcTs) suggested that ST6GalNAc1 may be responsible for the biosynthesis of previously identified sialyl-core 5 and sialyl-core 8. Surprisingly, our results indicated that ST6GalNAc4, 5, 6 also possess weak α2-3sialylstransferase activity. In conclusion, we provide efficient routes to access α-linked mucin glycan rare cores and their sialylated forms, which are indispensable probes and tools to study the biosynthesis and structure-function relationships of mucin glycans.

(P194) *TMEM260* encodes a protein-specific O-mannosyltransferase in mammals

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Mutations in the *TMEM260* gene cause structural heart defects and renal anomalies syndrome (SHDRA), but the function of the encoded protein has remained unknown. We identify TMEM260 as an ER-located protein O-mannosyltransferase that selectively glycosylates extracellular immunoglobulin, plexin, transcription factor (IPT) domains of the hepatocyte growth factor receptor (cMET), macrophage-stimulating protein receptor (RON), and plexin receptors. We find that disease-causing *TMEM260* mutations impair O-mannosylation of IPT domains and that TMEM260 knock out in cells results in receptor maturation defects and abnormal growth of 3D cell models.

Our discovery thus demonstrates that initiation of protein O-mannosylation is controlled by at least three distinct pathways (*POMT1/POMT2*, *TMTC1-4* and *TMEM260*) in mammals and that the receptor-specific O-Mannosyltransferase TMEM260 serves important biological functions during development.

(P195) Cell surface glycoconjugates recognized by cholera toxin

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Cholera toxin (CT) is an AB5 toxin produced by the bacterium Vibrio cholerae and is the causative agent of the disease cholera. Structural analysis of the B subunit of cholera toxin (CTB) has revealed that the toxin has two distinct glycan binding pockets that allow it to recognize glycoconjugates displayed on the surface of host cells. One pocket binds the glycan portion of the ganglioside GM1 with high affinity while the second binding pocket recognizes fucosylated glycans with much lower affinity. To gain further insight into cell surface glycans recognized by cholera toxin, we performed a CRISPR knockout screen in Colo205 intestinal epithelial cells. Analysis of the results revealed that genes involved in fucosylation, sialylation, and glycolipid biosynthesis are important regulators of CTB binding to host cells. Further, genetic manipulation of these genes resulted in changes in toxin internalization and host cell intoxication. Additional genes identified in the screen were classified as regulators of glycosylation. This study provides further insight into the possible mechanisms of host cell intoxication by CT as well as revealing mechanisms by which cell surface glycosylation is orchestrated.

(P196) Mucin-glycans attenuate microbial virulence

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Mucus is a biological gel that lines all wet epithelia in the body, including the mouth, lungs, and digestive tracts, and has evolved to protect us from pathogenic invasion. Microbial pathogenesis in these mucosal systems, however, is often studied in mucus- free environments, which lack the geometric constraints and microbial interactions that are found in natural, three-dimensional mucus gels. To bridge this gap, my laboratory has developed model test systems based on purified mucin polymers, the major gel-forming constituents of the mucus barrier, and their glycans. We use this model to understand how the mucus barrier influences microbial virulence, and moreover, to elucidate strategies used by microbes to overcome the normal protective mucus barrier. I will discuss data showing that the mucin polymers, and specifically their associated glycans, have a significant impact on the physiological behavior of microbes, including surface attachment, quorum sensing, the expression of virulence genes, and biofilm formation. The picture is emerging that mucin glycans are key host players in the regulation of microbial virulence and underscores the untapped therapeutic opportunities found in these host-derived molecules.