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Metabolism, Cell Surface Organization, and Disease

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Abstract

Genetic information flows from DNA to macromolecular structures—the dominant force in the molecular organization of life. However, recent work suggests that metabolite availability to the hexosamine and Golgi N-glycosylation pathways exerts control over the assembly of macromolecular complexes on the cell surface, and in this capacity, acts upstream of signaling and gene expression. The structure and number of *N*-glycans per protein molecule cooperate to regulate lectin binding and thereby the distribution of glycoproteins at the cell surface. Congenital disorders of glycosylation provide insight as extreme hypomorphisms, whereas milder deficiencies may encompass many common chronic conditions including autoimmunity, metabolic syndrome, and aging.

Introduction

In the study of development and disease the levels of receptor ligands and their downstream signaling pathways have received considerable attention, but less clear are the determinants of receptor availability at the cell surface. A sizeable proportion of receptors and transporters are located in endomembrane compartments that exchange with the cell surface pools (Wiley and Burke, 2001). Certainly, the number of receptors at the cell surface is an important determinant of ligand-receptor complex formation that affects signal strength (Dennis et al., 2009; Gurdon and Bourillot, 2001). Many cytokine receptors and transporters are co-translationally modified with Asn (N)- and Ser/Thr (O)- linked oligosaccharides (glycans), and together with proteoglycans and glycolipids, form the glycocalyx, a ~100 nM wide macromolecular structure that dominates the cell surface. The remodeling of N-glycans in the Golgi is sensitive to metabolism and when combined with gene-encoded differences in N-glycan number per protein, glycoprotein concentrations at the cell surface can be differentially regulated according to their affinities for endogenous lectins (Lau et al., 2007). Herein, we posit that increasing metabolic regulation of Golgi N-glycan biosynthesis in

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metazoan plays a role in adaptive cellular systems in postnatal life. Applying this model to disease states, we review data from mice and human studies linking genetic and metabolic dysregulation of protein N-glycosylation in congenital disorders and complex traits such as autoimmunity, cancer, and metabolic syndrome.

Glycans and cell surface regulation

Cell surface transmembrane glycoproteins are localized to and exchanged between microdomains, including clathrin coated-pits, cholesterol-rich lipid rafts, adhesion junctions between cells, substratum focal adhesion, and as discussed here, the galectin lattice. The galectins are N-acetyllactosamine-binding proteins, and their major ligands are Golgi-remodeled N-glycans common to many glycoproteins at the cell surface (Patnaik et al., 2006). Galectins are redox-sensitive proteins produced in the cytosol, and released by a non-classical pathway where they first encounter glycoproteins in cargo vesicles (Delacour et al., 2007) and at the cell surface (Hughes, 1999). Galectins, as well as the siglecs and selectins lectin families are largely absent in unicellular eukaryotes (Drickamer and Fadden, 2002). The 14 human galectins have either 1 or 2 carbohydrate recognition domains and mediate glycoprotein cross-linking (Lee and Lee, 2000). The extended C-terminus of galectin-3 forms multimeric structures (up to pentamers) driven by increasing concentrations of multivalent glycoprotein ligands, resulting in a molecular “lattice” or microdomain with irregular geometry at the cell surface (Ahmad et al., 2003; Demetriou et al., 2001). However, galectins with different lattice-forming geometries may bind glycoprotein partners selectively based on the N-glycan orientation (Brewer et al., 2002).

Members of the epidermal growth factor receptor (EGFR) family have been used extensively to study trafficking between microdomains, and sensitivity to ligand. The extracellular domain of EGFR is restrained from self-association and activation, but modeling and experimental data implicates additional extrinsic mechanism of restraint (Klein et al., 2004). One of these is galectin binding to the N-glycans on EGFR, which slows lateral diffusion and loss to coated-pit endocytosis in mammary carcinoma cells (Lajoie et al., 2007; Partridge et al., 2004). In human glioblastomas, an activating mutation in EGFR deletes part of the extracellular domain (EGFRvIII), removing 4 of 12 N-glycan sites and increasing ligand-independent dimerization (Fernandes et al., 2001). Domain II–IV normally confers intramolecular interactions that maintain the autoinhibited state, where the N-glycans in domain III contribute by blocking receptor dimerization and activation (Takahashi et al., 2008) (Tsuda et al., 2000). Therefore, in addition to opposing loss to endocytosis, N-glycans appear to “insulate” against ligand-independent EGFR dimerization. N-glycans extend from the protein surface and may restrict the approaches between monomers for EGFR dimerization. Galectin cross-linking is expected to separate glycoproteins by ~100Å, possibly imposing a physical barrier to spontaneous dimerization (Seetharaman et al., 1998) (Figure 1). However, the lattice is not likely a barrier to ligand-mediated receptor dimerization, as various cyokine affinities for cognate receptors are ~10^{8}-10^{12} M, whereas galectins affinities for glycans are lower ~10^{-5}-10^{-7} M. Galectin-3 and -8 bind to β1 integrin and regulate focal adhesion turnover and cells motility (Lagana et al., 2006; Levy et al., 2001). As expected for a multivalent system, addition of recombinant galectin to cells displays an optimum for stimulation of focal adhesion turnover. Galectin gene expression, export and redox sensitivity are likely important aspects of lattice regulation and warrant further investigation.

The α and β subunits of the T cell receptor (TCR) are N-glycosylated on at least 7 Asn-X-Ser/Thr (NXS/T) sites and the complex is assembled in the endoplasmic reticulum (ER) prior to N-glycan processing in the Golgi (Rudd et al., 1999). On naive T cells, galectin-3 binds to the T cell receptor (TCR) and opposes microfilament-dependent partitioning of

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TCR into lipid microdomains (ganglioside GM1-enriched), while blocking microfilament-dependent movement of the tyrosine phosphatase CD45 out of the lipid microdomains (Chen et al., 2007; Demetriou et al., 2001) (Figure 1). CD45 in lipid microdomains dampens TCR-Lck signaling. Galectin binding to TCR impedes CD8 binding, another example of insulating against complex formation that increases the threshold to TCR agonists (Demotte et al., 2008). Mice deficient in the branching enzyme β1,6-N-acetylglucosaminyltransferaseV (Mgat5) or β1,3-N-acetylglucosaminyltransferase (β3GnT2), an enzyme that extends the β1,6 branches with poly-N-acetyllactosamine, drops the barrier to TCR clustering and autoimmune disease *in vivo* (Demetriou et al., 2001; Togayachi et al., 2007). Selective removal of specific NXS/T sites from TCR also enhances receptor diffusion, multimerization, and activation (Kuball et al., 2009). T cell activation results in adaptive changes in glycoprotein distribution at the surface downstream of increased nutrient uptake, membrane turnover, and N-glycan branching.

Similarly, oncogenesis stimulates membrane remodeling, N-glycan branching and galectin-3 expression (Dennis et al., 1987; Lanzetti et al., 2004; Takenaka et al., 2004). Therefore tension appears to increase between membrane turnover and lattice-mediated protection of receptors from coated-pits and loss to endocytosis (Figure 1). Cancer mutations that disrupt the endocytic machinery also protect growth receptors either at the cell surface or by maintaining their activity in endosomes (Mosesson et al., 2008). For example, *Avalanche* and *rab5* mutants in the *Drosophila* endocytic pathway inhibit internalization of surface receptors to the early endosome; thus an excess of surface growth receptors promotes growth signaling (Bilder, 2004). However, the scrib-dlg-lgl tumor suppressor pathway also disrupts trafficking, and thereby signaling in endosomes by tumor necrosis factor (TNF)/JNK to mediate cell death (Igaki et al., 2009). Protein phosphatases are important constitutive suppressers of growth signaling in early endosomes and lipid microdomains (Reynolds et al., 2003). The ligands for receptor tyrosine phosphatase have been elusive, and regulation may involve galectins as has been suggested for CD45 (Chen et al., 2007) (Figure 1).

In addition to coated-pit endocytosis and the galectin lattice, receptors exchange with other specialized membrane microdomains. For example, EGFR is tethered by epithelial cell junctions where it binds to the tumor suppressor neurofibromatosis (NF2/Merlin), which reduces receptor sensitivity to EGF (Curto et al., 2007). Adhesion junctions are lost with cancer progression, in a process known as the epithelial to mesenchymal transistion (EMT). EMT promotes actin remodeling, which stimulates increased trafficking of EGFR between endosomes and the cell surface. As a consequence, the ruffling edges of cells recruit and activate EGFR with lower ligand requirements (Moro et al., 2002).

Caveolin1 (Cav1) clusters in cholesterol-rich rafts and binds to conserved motifs found in EGFR and other signaling proteins resulting in loss of responsiveness to growth factors (Okamoto et al., 1998). CAV1 maps to a tumor suppressor locus (D7S522; 7q31.1) (Lee et al., 2002), but up-regulation of N-glycan branching in mouse mammary tumor cells acts dominantly to protect EGFR from Cav1 suppression (Lajoie et al., 2007). The galectin lattice protects EGFR from loss to Cav1 microdomains (Lajoie et al., 2007). Cav-1 binds cholesterol in rafts and stabilizes the structure by reducing diffusion, and interestingly, galectin-4 and -9 binding to glycoproteins in lipid rafts has a stabilizing effect (Braccia et al., 2003; Tanikawa et al., 2008). As outlined below, both N-glycan number (sequence-encoded NXS/T) and Golgi N-glycan branching (context-dependent) determine glycoprotein affinities for the lattice (Lau et al., 2007).

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N-glycoform diversity

N-glycosylation of proteins begins with the co-translational transfer of $Glc_3Man_9GlcNAc_2$ to ~ 70% of lumenal N-X-S/T (where X≠Pro) motifs by oligosaccharyltransferase (Apweiler et al., 1999). N-glycosylation is enhanced by neighboring aromatic amino acids and turns that expose the motifs at the convex surface of the peptide, but less efficient in the proximity of disulfide bonds, the transmembrane domain, and at the termini of protein (Jones et al., 2005). After transfer and trimming of 2 glucose (Glc) units, the protein chaperones calnexin and calreticulin bind to $Glc_1Man_9GlcNAc_2$ and the remaining Glc residue is recycled by α-glucosidase II and ER α-glucosyltransferase until folding is complete, allowing transit to the Golgi (Helenius and Aebi, 2004). However, most NXS/T sites are not required for protein folding, and site densities in most animals glycoproteins is higher than expected based on sequence composition, consistent with a post-Golgi function at the cell surface.

Chaperone-assisted protein folding employs an ancient and relatively homogenous N-glycan structure required prior to the Golgi. In contrast, an explosion in structural diversity has occurred with metazoan evolution of the Golgi pathways. Mannose residues are removed in the *cis* Golgi, then the N-acetylglucosaminyltransferases I, II, III IV, V (encoded by *Mgat1*, *Mgat2*, *Mgat3*, *Mgat4a/b*, and *Mgat5*) initiate the branches in a sequential pathway (Schachter, 1986) (Figure 1). Differing from the other Mgat enzymes, the N-acetylglucosamine (GlcNAc) added to the β-linked mannose residue by Mgat3 is not sequential nor elongated with galactose, and can block further branching by Mgat2, 4 and 5. In the *trans* Golgi, β1,4 galactosyltransferase catalyzes the addition of galactose to GlcNAc residues to generate the N-acetyllactosamine units, the epitope for galectin binding. The β1,6GlcNAc-branched product of Mgat5 is the preferred acceptor for extension with additional N-acetyllactosamine units, which increases affinity for galectins-1, -3, -8, and -9 (Hirabayashi et al., 2002). For example, galectin-3 binds to mono-, bi-, tri-, tetra-antennary N-glycans, and tetra-antennary N-glycans with polyN-acetyllactosamine with K_d's of 4.0, 1.4, 0.78, 0.69 and >0.19 μM, respectively, a synergistic increase with respect to N-acetyllactosamine units (Galβ1,4GlcNAcβ1,3). Importantly, structural diversity is magnified by partial saturation of branching at each N-glycan, as well as variable termination with sialic acid (SA), fucose (Fuc) and/or N-acetylgalactosamine (GalNAc). Terminal α2,6SA reduces N-glycan affinity for galectins, whereas GalNAc addition may increase affinities (Amano et al., 2003; Stowell et al., 2008).

In theory the diversity of the polymerization of monosaccharides into branched chains can increase in a factorial manner. However, the potential for N-glycan structural diversity in animal tissues is bounded by the specificities of Golgi enzymes encoded in animal genomes and their tissue-specific patterns of gene expression. Nonetheless, more than 40 structures (variations on branching and extension) have been identified on EGFR (Stroop et al., 2000). The protein environment of NXS/T sites can limit the accessibility to branching enzymes (Do et al., 1994), but even with some site-specific bias considerable numbers of glycoforms are possible (Figure 2). For example, 8 NXS/T sites in EGFR are occupied with N-glycans, and if we use a conservative estimate of 14 possible structures at each site, the potential number of glycoforms is 203,490, ~10 times the number of surface EGFR molecules per cell (Lau et al., 2007). Glycoproteins differ in encoded NXS/T number, and this should influence the requirements of Golgi remodeling as a determinant of lattice avidity and surface retention.

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NXS/T multiplicity encodes lattice affinity

The incidence of canonical N-glycosylation sequons (NXS/T, where X≠P) varies considerably between different glycoproteins. Receptor kinases that stimulate growth and proliferation such as insulin receptor, EGFR, and platelet derived growth factor receptor (PDGFR)] have ~5 times more NXS/T sites, longer extracellular domains and more sites per 100 amino acids than receptors that mediate organogenesis, differentiation and arrest (such as Tie1, Musk, Ltk, ROR1/2, DDR1, TβR, and EphR) (Lau et al., 2007). These observations suggest that N-glycan numbers per peptide (multiplicity) is a conserved and functionally significant feature of receptor kinases. NXS/T sites are the primary determinant in N-glycan multiplicity, and together with N-glycan branching determine glycoform structures and affinity for the galectin lattice. through. Membrane glycoproteins with only one or two N-glycans per peptide are markedly dependent on branching for stable association with the galectin lattice (Lau et al., 2007). Examples of low multiplicity glycoproteins shown to be dependent on branching are discussed below and include glucose transporter (GLUT4, GLUT2), cytotoxic T lymphocyte antigen–4 (CTLA-4), and TGF-β receptors (TβR).

Interaction of metabolism with N-glycan number and diversity

Fructore-6-P, glutamine and acetyl-CoA are central to carbohydrate, nitrogen, and fatty acid metabolism, and also required for *de novo* uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) biosynthesis by hexosamine pathway (Grigorian et al., 2007). The Golgi UDP-GlcNAc antiporter exchanges uridine monophosphate (UMP) for UDP-GlcNAc and establishes the steady state amounts of UDP-GlcNAc inside the Golgi. Although difficult to measure, estimates suggest that UDP-GlcNAc concentration is 15-fold higher in the Golgi (~1.5mM) than in the cytoplasm (Waldman and Rudnick, 1990). UDP-GlcNAc levels are well above the K_m for Mgat1 at 0.04 mM, but Mgat1 has a relatively low affinity for acceptor N-glycan at ~2 mM (Figure 1). This relationship is reversed for Mgat4 and Mgat5 enzymes, where UDP-GlcNAc concentrations become limiting (Lau et al., 2007; Sasai et al., 2002). In other words, Mgat1 and Mgat2 activities are suboptimal and limited by affinity for acceptors, whereas Mgat4 and Mgat5 activities are limited by UDP-GlcNAc concentration. Thus, the degree of N-glycan branching is dependent on rates of bulk protein synthesis, notably 5′cap-dependent mRNA translation stimulated by mTor/S6 kinase signaling, as well as the availability of key metabolites to UDP-GlcNAc biosynthesis. As a result, the high affinity galectin ligands, the tri- and tetra- antennary N-glycans, increase in an ultrasensitive manner as a function of UDP-GlcNAc availability (Lau et al., 2007). Ultrasensitivity describes a stimulus/response relationship in which the response rises sharply over a narrow range of stimulus (a sigmoid curve) with a Hill coefficient (nH) ≫1 indicating the deviation from a graded (hyperbolic) response curve (Ferrell, 1996). These pathway dynamics result from decreasing Mgat1, 2, 4, 5 activities moving down the pathway, decreasing affinities for the common substrate (K_m for UDP-GlcNAc), sequential order, and limited reaction times due to transit of glycoprotein substrates through the Golgi (Lau et al., 2007).

TCR and growth-promoting receptor tyrosine kinases (such EGFR, insulin receptor, PDGFR) where N-glycan multiplicity is relatively high, a significant fraction of receptors are associated with the lattice, and only a slow graded increase occurs with rising UDP-GlcNAc (1.5 to 6 mM). In contrast, glycoproteins with 1 or 2 N-glycans (such as TβR, CTLA-4, and GLUT4) are largely below the threshold for stable association with the galectin lattice, but increase in a switch-like (sigmoidal) response to increasing UDP-GlcNAc concentration, mirroring the ultrasensitive output of tri- and tetra-antennary N-glycans by the branching pathway. Experimental data and computational modeling are consistent with this model for the retention of glycoproteins on the cell surface. Their half-lives are proportional to avidities for the lattice and cell surface retention is countered by

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constitutive endocytosis (Lau et al., 2007). To summarize, ultrasensitivity of the N-glycan branching pathway to UDP-GlcNAc allows differential regulation of surface glycoproteins based on N-glycan number and metabolite flux. Thus, metabolic supply to the hexosamine pathway titrates glycoprotein expression at the cell surface with response dynamics characteristic of N-glycan number.

Consequences of N-glycan number

Receptor kinases and cancer

In non-transformed epithelial cells, surface EGFR, PDGF, IGFR first increase in a shallow graded manner with increasing UDP-GlcNAc, followed by TβR in a switch-like response. These dynamics of receptors regulation by hexosamine/Golgi/ lattice have features of an oscillating system that may support tissue homeostasis (Lau et al., 2007). The same stimulus (UDP-GlcNAc) promotes a Michaelis-Menten response and a sigmoidal response for high and low multiplicity receptors, respectively. The intervening delay allows growth signaling and anabolic metabolism to prevail before the onset of negative regulation as UDP-GlcNAc rises to levels that retain the low multiplicity receptor. The TβR and receptor tyrosine kinase pathways often oppose each other in proliferation and differentiation. For example, self-renewal and pluripotency of cultured human embryonic stem cells is maintained by a precise ratio of fibroblast growth factor (bFGF) to Noggin, a bone morphogenic protein (BMP)/ TGF-β antagonist (Xu et al., 2005). Similarly, BMP4 promotes the differentiation of tumor-initiating stem cells and inhibits growth of human glioblastoma cells in mice (Piccirillo et al., 2006). Canonical TβR/SMAD2/3 signaling also slows cell cycle progression by suppressing c-myc expression (Matsuura et al., 2004; Seoane et al., 2004). c-Myc activity in tumors stimulates the uptake of glucose and glutamine, the major carbon sources for energy in cancer cells (DeBerardinis et al., 2008), and stimulates anabolic metabolism including UDP-GlcNAc supply (DeBerardinis et al., 2008; Morrish et al., 2009). Low multiplicity of TβR and membrane remodeling in cancer cells reduces its surface levels. Although Raf/ Mak/Ets activation increases Mgat4 and Mgat5 gene expression (Buckhaults et al., 1997; Ishida et al., 2005; Takamatsu et al., 1999), this appears to be insufficient to protect surface TβR, and conditions in cancer cells favor high multiplicity growth receptors over TβR (Lau et al., 2007). Metabolic supplements to UDP-GlcNAc can restore TβR signaling and slow growth suggesting that with a better understanding of N-glycosylation and metabolism, it may be possible to restore a “non-transformed” distribution of surface glycoproteins (Lau and Dennis, 2008; Mendelsohn et al., 2007).

T cell activation

T cell activation and subsequent arrest/apoptosis are regulated by gene expression, but are also temporally affected by metabolism and environment cues (Alegre et al., 2001; Frauwirth et al., 2002). Activation of naïve T cells requires TCR clustering above a threshold number, which induces Erk/PI3K signaling, glucose uptake and multiple rounds of cell division. The interval until growth arrest 4-5 days later is determined in part by stimulation of metabolite flux into UDP-GlcNAc supply (Lau et al., 2007). Similar to TβR, CTLA-4 has 2 NXS/T sites and displays an ultrasensitive response to UDP-GlcNAc to attain the branching required for surface retention and growth arrest (Lau et al., 2007). The levels of CTLA-4 transcript and intracellular protein increase markedly with TCR signaling during the early growth phase (Chikuma and Bluestone, 2002), but endosomal trafficking limits expression of CTLA-4 at the cell surface, keeping it below the threshold for the induction of growth arrest until the avidity of CTLA-4 for the galectin lattice increases. Surface CTLA-4 promotes T cell motility, which dampens TCR-CD28 signaling in blast cells leading to quiescent memory cell status or apoptosis (Schneider et al., 2006). T cells deficient in Mgat5 are hypomorphic for CTLA-4 at the cell surface but not inside the cells, and surface levels

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can be rescued by supplementation to UDP-GlcNAc as well as via maximal CD28 co-stimulation which drives metabolism. Compensation for the absence of tetra-antennary glycans in Mgat5 deficient cells occurs by supplementation to early reactions leading to more tri-antennary N-glycans. Galectin-1-deficient mice are also susceptibility to autoimmune disease, and both galectins-1 and -3 play a role in T cells apoptosis (Stillman et al., 2006; Toscano et al., 2007).

Glucose transporters

The glucose transporters GLUT2 and GLUT4, each with a single N-glycan, are dependent on branching and galectin binding for optimal surface retention (Lau et al., 2007; Ohtsubo et al., 2005). Insulin receptor (INSR) has 18 potential sites of N-glycan attachment, thus a large disparity with the GLUTs, and conducive to differential regulation by UDP-GlcNAc. INSR stimulates glucose uptake and surface GLUT4 in muscle with a response described as “quantum” (ultrasensitive), and this effectively shuttles excess serum glucose into muscle (Coster et al., 2004). Supplementation to the hexosamine pathway increases surface GLUT4 in a sharp sigmoidal response, and mutation of the GLUT4 NXS/T site blocks the response to UDP-GlcNAc. GLUT4 transgenic mice display increased UDP-GlcNAc levels in muscle, indicating positive feedback to the hexosamine pathway (Buse et al., 1996). Metabolites can change very rapidly in cells regulated largely by allosteric mechanisms (Bailey, 1998), but the hexosamine/Golgi/lattice may adapt the cell surface to reflect conditions averaged over time. In this regard, diabetic hyperglycemia leads to TGF-β-dependent tissue fibrosis, possibly due to a hexosamine-dependent increase in surface retention of TβR (Kolm-Litty et al., 1998).

The hexosamine pathway

UDP-GlcNAc is required in the biosynthesis of most classes of extracellular glycopolymers, including the N- and O-glycans, glycolipids, chitin, hyluronic acid, and heparin sulfate proteoglycans. UDP-GlcNAc is essential in all three phylogenetic kingdoms, and the *de novo* synthesis pathway may have originated with passage of life through the “RNA world”. As a vestige of this heritage, RNA transcripts for glutamine:fructose-6P aminotransferase (GFAT) in gram-negative bacteria have a 5′ aptomer that binds GlcNAc for allosteric control of its ribozyme activity. GlcNAc binding induces cleavage of the message and downregulates GFAT, which is a rate-limiting enzyme of the pathway (Winkler et al., 2004). GFAT catalyzes isomerase and amination reactions using glutamine as the amine donor, and the enzyme itself is allosterically inhibited by its product glucosamine-6-phosphate (GlcN-6P) and by UDP-GlcNAc in mammalian cells (Broschat et al., 2002). The substrates of the *de novo* pathway, fructose-6P, glutamine and acetyl-CoA are highly regulated intermediates of carbon and nitrogen metabolism (Figure 3).

In mammalian cells, N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) are salvaged into the hexosamine pathway from glycoconjugate turnover by hydrolysis of glycosidic linkages in lysosomes and transported into the cytoplasm by uncharacterized transporter(s), where they are phosphorylated and re-enter the UDP-GlcNAc pool. There are two routes for salvage of GlcN-6P, either acetylation to GlcNAc-6P by glucosamine-6P N-acetyltransferase GNPAT1/Emeg32 (Boehmelt et al., 2000) and UDP-GlcNAc production, or deamination and conversion to fructose-6P by glucosamine-6-phosphate isomerase/oscillin (GNPDA) and entry into glycolysis (Zhang et al., 2003) (Figure 3). Feedback inhibition of GFAT by GlcN-6P with possible activation of GNPDA may convert most GlcN to fructose-6P. In contrast, the product of GlcNAc-6-kinase (NAGK), is transferred directly into UDP-GlcNAc as there appears to be little feedback inhibition beyond GNPAT1 (Grigorian et al., 2007). Thus, GlcNAc is spared from catabolism, efficiently salvaged, and contributes to anabolic pathways of glycoprotein modification in cultured mammalian cells.

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UDP-GlcNAc is a precursor to cytidine monophosphate-sialic acid (CMP-SA), the sugar-nucleotide donor utilized by siayltransferases to cap N- and O-glycans (Figure 3). Loss-of-function mutations in the gene encoding UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE/MNK) results in an autosomal recessive neuromuscular disorder in humans and early death in mice (Hinderlich et al., 1997). Supplementation of pregnant mice with ManNAc prolongs the survival of the *Gne* mutant pups (Galeano et al., 2007), further supporting a role for amino-sugars as therapeutics.

O-GlcNAc transferase (OGT) adds O-GlcNAc to Ser/Thr residues on many cytosolic proteins, and removal by O-GlcNAcase results in rapid turnover (Kreppel and Hart, 1999). OGT overexpression in liver or fat promotes insulin resistance and hyperleptinemia in mice (McClain et al., 2002). O-GlcNAc addition to hepatic FoxO1 promotes the expression of genes for gluconeogenesis (Housley et al., 2008). Similarly, OGT competes at AMPK phosphorylation sites in CRTC2 allowing nuclear translocation and transcription of gluconeogenesis genes (Dentin et al., 2008). Glucose starvation activates AMPK and paradoxically, also up-regulates OGT gene expression in cultured HepG2 hepatocellular carcinoma cells (Taylor et al., 2008). Recently, *Drosophila* super sex combs (Sxc/OGT) has been shown to be a member of the Polycomb group (PcG), and mutation of Sxc provide strong evidence that O-GlcNAc is required for transcriptional repression of homeobox (HOX) proteins (Gambetta et al., 2009). N-glycan branching regulates glucose homeostasis in whole animal studies (Cheung et al., 2007; Ohtsubo et al., 2005), and further work is required to determine how OGT, N-glycan branching and the hexosamine pathway interact.

N-glycan branching in evolution and development

Phylogenetics provides insight into the role of an ultrasensitive N-glycan branching pathway. Most mature N-glycans in *Arabidopsis*, *C. elegans*, and *Drosophila* are mannose-terminated structures, and compared to mammals, very little branching occurs beyond mono- and a trace of bi-antennary (Zhu et al., 2004). Curiously, β-N-acetylhexosaminidase activity in these species specifically removes most of the GlcNAc residues added by Mgat1, and this blocks further branching (Gutternigg et al., 2007). Considering enzyme affinities for UDP-GlcNAc, the levels of mono-antennary N-glycans may depend largely on competition between Mgat1 and β-hexosaminidase, whereas the supply of UDP-GlcNAc may regulate the formation of bi-anntennary N-glycans by Mgat2 (K_m ~1 mM for Mgat2 and <0.1 mM for Mgat1) (Figure 1). N-glycan branching in *C. elegans* and *Drosophila* may be more dependent on developmental regulation of Golgi enzymes and less on UDP-GlcNAc metabolism (Figure 4). Furthermore, Golgi β-N-acetylhexosaminidase activity is not found in mammalian cells. This corresponds with the evolutionary expansion of the branching pathway in vertebrates (which have more *Mgat* genes) generating higher affinity galectin ligands. As described above, these features are key to differential regulation of glycoprotein with high verses low numbers of N-glycan attachment sites. Most receptor kinases in *C. elegans* and *Drosophila* have high NXS/T multiplicity, and interestingly, vertebrates show an expansion of genes encoding receptors with lower multiplicity (Lau et al., 2007). Notably these are TGF-β/BMP, Eph receptors, and some classes of receptor tyrosine kinases. Evolutionary trends in N-glycan branching and NXS/T multiplicity may be due to selection pressures for conditional control of growth and the adaptive immune system in longer-lived animals.

Mouse embryos lacking all N-glycan branches due to Mgat1 deficiency die by E9.5 with defects in growth and morphogenesis (Ioffe and Stanley, 1994; Metzler et al., 1994) (Figure 4). Mgat2 initiates the second antenna and displays an absolute requirement for the prior action of Mgat1 and α-Mannosidase II, consistent with sequential evolution and regulation of the pathway described above. Human Mgat2 deficiency (CDG-IIa) (Tan et al., 1996) and

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mice lacking Mgat2 (Wang et al., 2001) display similar developmental and postnatal defects. Mice with mutations in genes encoding enzymes further down the branching pathway are viable, but display postnatal defects in the immune system, metabolism, tissue renewal, and delays in cancer development. Mgat4a and Mgat5 both generate galectin ligands, but have opposing effects on systemic glucose homeostasis in gene knockout studies. Mgat4a is required for tri- and tetra- antennary N-glycans in pancreatic β cells, whereas Mgat5 generates tetra-antennary branches and is expressed more widely. Mgat5-deficient mice are resistant to weight gain on an enriched diet, are hypersensitive to fasting, and are mildly hypoglycemic (Cheung et al., 2007). In contrast, mice deficient in Mgat4a display hyperglycemia, obesity, and insulin insufficiency on an enriched diet (Ohtsubo et al., 2005). Mgat4a is required in the modification of the single N-glycan chain on GLUT2, which binds to galectins and promotes cell surface retention. Elevated GLUT2 increases glucose uptake in β cells, acting as a stimulus for insulin secretion at peak serum glucose. Insulin responses appear normal in Mgat5 deficient mice, but changes in GLUT4 surface retention in muscle and fat coupled with impaired glucagon responses in liver may contribute to the lean phenotype (Cheung et al., 2007). Surface expression of glucagon receptor and cAMP signaling are deficient in hepatocytes from $Mgat5^{-/-}$ mice, which may contribute to these phenotypes. Resistance to weight-gain in Mgat5 deficient mice is rescued by supplementing their drinking water with GlcNAc but not GlcN (A. Johswich, M. Ryczko, J. Dennis, unpublished data).

Muscle satellite cells and bone marrow cells from Mgat5 deficient mice display an imbalance favors Smad2/3 to Erk signaling, accompanied by an increased ratio of differentiation to renewal. *In vivo*, bone marrow osteoprogenitors and muscle satellite cells are depleted and the normal aging processes in bone and muscle of Mgat5 deficient mice is accelerated (Cheung et al., 2007). Stem cell maintenance requires trophic factors and surface levels of cognate receptors, which both decline with normal aging (Conboy et al., 2005; Shiraha et al., 2000). It is possible that intervention directed at hexosamine and other metabolic pathways might support the “youthful” distributions of surface glycoproteins that more effectively support stem cell renewal. Profiling or proteomics of the cell surface by mass spectrometry has the potential to reveal critical changes in receptor and transporters (Wollscheid et al., 2009). The Klotho gene was identified as a senescence-suppressor, and encoding a putative α2,6 sialidase that enhances N-glycan affinity for galectin-1 and protects glycoproteins from coated-pit endocytosis. Loss of Klotho results in increased retention of the surface renal Ca^{2+} and K^{+} channels TRPV5 and ROMK1, each with one available NXS/T site (Cha et al., 2009; Cha et al., 2008). Adaptation of the cell surface by metabolic flux to Golgi N-glycosylation in youth may buffer against environmental stresses and genetic variation, but decline with aging due to a shift from glycolytic to oxidative metabolism (Petersen et al., 2003). Thus life history and genetic background may interact through N-glycan-dependent regulation of glycoproteins to reveal late-onset diseases such as autoimmunity, metabolic disease, and cancer.

Congenital Disorders of Glycosylation

Congenital disorders of glycosylation (CDG) are hypomorphic defects in N-glycan biosynthesis, and provide insight into the functions of N-glycans in humans. Type I CDGs are deficiencies in various enzymes in the biosynthetic pathway for $Glc_3Man_9GlcNAc_2$-pp-dolichol, the donor for N-glycan addition. As a result, in CDG usage of NXS/T sites is reduced (reviewed in (Jaeken and Matthijs, 2007) (Figure 2).

CDG-Ib is a deficiency in phosphomannose isomerase (PMI, Figure 3) that decreases the supply of guanosine diphosphate mannose (GDP-Man) for dolichol-pp-oligosaccharide synthesis. Unlike most CDGs, the CDG-Ib defect can be treated with a monosaccharide supplement that alleviates the chronic postnatal phenotypes (Freeze, 1998). Failure to thrive,

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coagulopathies, protein-losing enteropathy, and liver fibrosis observed in CDG-Ib are improved by supplementing the diet with mannose, which bypasses the defective step (conversion of Fru-6P to Man-6P) and increases Man-6P and GDP-Man by the action of hexokinase. Glucose deprivation in cultured cells causes reduced glycosylation of sites in EGFR and altered distribution at the plasma membrane (Konishi and Berk, 2003). Low glucose conditions impairs $Glc_3Man_9GlcNAc_2$ –pp-dol biosynthesis giving rise to smaller intermediates and incomplete N-glycosylation. The resulting unfolded protein response activates PERK, which inhibits general translation by phosphorylating the translation initiation factor eIF2α, and this allows Glc3Man9GlcNAc2 –pp-dol and N-glycosylation levels to recover (Shang et al., 2007). Fibroblast from patients with type I CDG displayed similar but moderate induction of PERK kinase, suggesting that ER and metabolic stress may be a common feature of CDG-I (Lecca et al., 2005).

Subjects with defects in the Golgi and secretory pathway are classified as having type II CDG, which is characterized by reduced branching or extension to the N-glycans (Figure 2). The clinical phenotypes of type I and II CDG are often similar. In addition to those clinical features noted above, features of type II CDG include psychomotor retardation, ataxia, seizures, retinopathy, and dysmorphic features. These deficiencies arise from loss-of-function mutations in genes related to remodeling; notably *Mgat2* (CDG-IIa), *α-glucosidase I* (CDG-IIb), *GDP-Fucose Golgi transporter* (CDG-IIc), and *β1,4-galactosyltransferase* (CDG-IId) (Jaeken and Matthijs, 2007). Leukocyte adhesion deficiency type II (LAD II or CDG-IId), is caused by a hypomorphic mutation in the Golgi GDP-Fuc transporter that reduces cell surface fucosylated glycans and patients suffer recurrent infections due to a deficiency in selectin ligands. The fucose salvage pathway has the capacity to generate very high concentrations of GDP-Fuc, sufficient to overcome a partial defect in Golgi GDP-fucose transport in patients (Marquardt et al., 1999) (Figure 3). Similarly, fucose fed to mice deficient in GDP-keto-4-keto-6-deoxymannose 3,5-epimerase-4-reductase (FX), restores fucose content in glycoproteins (Smith et al., 2002). Although the developmental defects associated with CDG are irreversible, postnatal defects can in some cases be rescued by dietary supplements to sugar-nucleotide pools. The similarities in CDG clinical phenotypes suggest that NXS/T site usage (CDG type I) and Golgi N-glycan remodeling (CDG type II) are largely convergent for glycoprotein localization at the cell surface and in matrix.

Glycosylation defects in complex human diseases

Metabolism is embedded with multiple feedback mechanisms, but homeostatic set-points for glucose and fatty acid metabolism change with aging in manner that is poorly understood (Petersen et al., 2003). Autoimmune disease and sporadic cancers are examples of diseases with stochastic etiologies caused by one or a few cells expanding in a pathological manner (Dennis et al., 2002). Therefore, small changes in homeostasis can be clinically important in large cell populations, given sufficient time and external stresses.

CTLA-4 has low N-glycan multiplicity, is subject to high rates of constitutive endocytosis (Alegre et al., 2001), and requires the galectin lattice to promote T cell growth arrest (Lau et al., 2007). A common polymorphism in CTLA-4 (T17A) reduces N-glycan occupancy at the two NXS/T sites by ~50%, and diminishes surface expression (Anjos et al., 2002). Although CTLA-4-deficient mice display widespread autoimmunity (Waterhouse et al., 1995), CTLA-4(Ala17) alone contributes little risk to multiple sclerosis in humans. However, the addition of fewer N-glycans can be viewed as a selective “CDG type I deficiency” with the potential to promote disease in combination with Golgi polymorphisms (“mild CDG type II deficiency”). Indeed, polymorphisms in human Mgat1 alter branching and interact with CTLA-4(Ala17), and coinheritance cooperatively suppresses CTLA-4 surface expression and synergistically increases the risk of multiple sclerosis, with neither variant promoting

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disease in the absence of the other (H.-L. Chen, J. Dennis, M. Demetriou unpublished data). Importantly, both TCR sensitivity and defects in CTLA-4 surface expression are conditional on UDP-GlcNAc supply to the N-glycan branching pathway and rescued by supplementation with GlcNAc. Dietary supplements of GlcNAc also suppress autoimmune diabetes in non-obese diabetic mice (Grigorian et al., 2007), and chronic inflammatory bowel disease in children (Salvatore et al., 2000).

Interestingly, several mouse strains, including PL/J mice, are prone to autoimmunity and are naturally hypomorphic for GlcNAc branching, with deficiencies in Mgat1, Mgat2 and Mgat5 enzyme activities (Lee et al., 2007). PL/J mice are also metabolic outliers among mouse strains displaying features similar to Mgat5-deficient mice on a 129 or C57B6 background, notably small size, resistance to a high fat diet, low respiratory quotient, and shorter lifespan. In addition to genetics, nutrient conditions during embryogenesis are known to affect skeletal development as well as metabolism later in life. A poor nutrient environment in utero appears to adapt the metabolic circuitry for harsh conditions in postnatal life (Gluckman and Hanson, 2006). Poor human prenatal nutrition coupled with the high-calorie diet readily available in industrialized nations may predispose to high body mass and diabetes (Bateson et al., 2004). Perhaps nutrient supply to the fetus or newborn influences the homeostatic set-point for hexosamine/Golgi/lattice and qualitative aspects of extracellular matrix, acting as a metabolic and self-organizing regulatory network in post-natal life.

Emerging evidence also links N-glycan branching to specific functions in the nervous system. Mgat5 is also highly expressed in the central nervous system (Granovsky et al., 1995), and mice deficient in Mgat5 display resistance to depression-like behavior in response to stress (Soleimani et al., 2007). In most sporadic amyotrophic lateral sclerosis (ALS) patients, EAAT2 protein and transport activity are reduced by 30-90%, which is not readily explained by mRNA levels. However, an allele of *EAAT2* with a point mutation that removes one of two N-glycosylation sites suggests surface expression is dependent on N-glycans, possibly because of retention by lectins (Aoki et al., 1998; Trotti et al., 2001). Oxygen radical stress is a prominent feature of ALS that may also disrupt the lattice by oxidation of galectins.

Concluding remarks

Kuriyan and Eisenberg (2007) argue that an early driving force in natural selection was low-affinity colocalization of proteins that enhance mass action, and subsequent evolution of many variations on high affinity and allosteric control of protein networks. Structurally, the galectin lattice is a force for localization and organization of surface glycoproteins, but in an evolutionary sense, lectin-carbohydrate lattices may be tolerant to variation in structural details, and still maintain essential parameters of the regulatory network. Lectins bind with low monomeric affinities, but multivalency allows a very wide range of affinities, and provides flexibility for evolutionary insertion of new glycan structures. From a chemist's view, the N-glycan pathway might appear to be a very imprecise process, essentially imposing “entropy” to the interface of cells with their environment. However, in biological systems, variance is tolerated and often serves a feedback regulatory role at the network level (Coffey et al., 1998; Maamar et al., 2007; Radman, 2001). Defining all glycoforms in time and space is obviously an unattainable goal, and not likely to make a meaningful contribution to our understanding of cell behavior. Rather, glycoforms can be classified in terms of their affinity for the animal lectins and as part of a larger network that regulates membrane organization.

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Finally, gene-gene and gene-environmental interactions underpin many complex trait diseases. As such, genome-wide screens in complex diseases such as type 2 diabetes, autoimmunity and cardiovascular disease, generally find individual single nucleotide polymorphisms that account for very modest risk (<1.5) and therefore only a small fraction of the total genetic risk for the disease in question (Manolio et al., 2009). Cell surface glycoproteins regulate highly adaptive systems in animals, and their regulation in a conditional manner should permit heritable phenotypic variation, evolutionary fitness, and opportunities for radiation. However, greater adaptive potential may also come with greater risk of polygenic diseases and proliferative disorders such as cancer and autoimmune disease. We suggest that genetic variations in hexosamine/N-glycan pathways and substrate glycoproteins interact with each other and with life-history changes in metabolism to account for a large portion of “missing” risk for many chronic human diseases.

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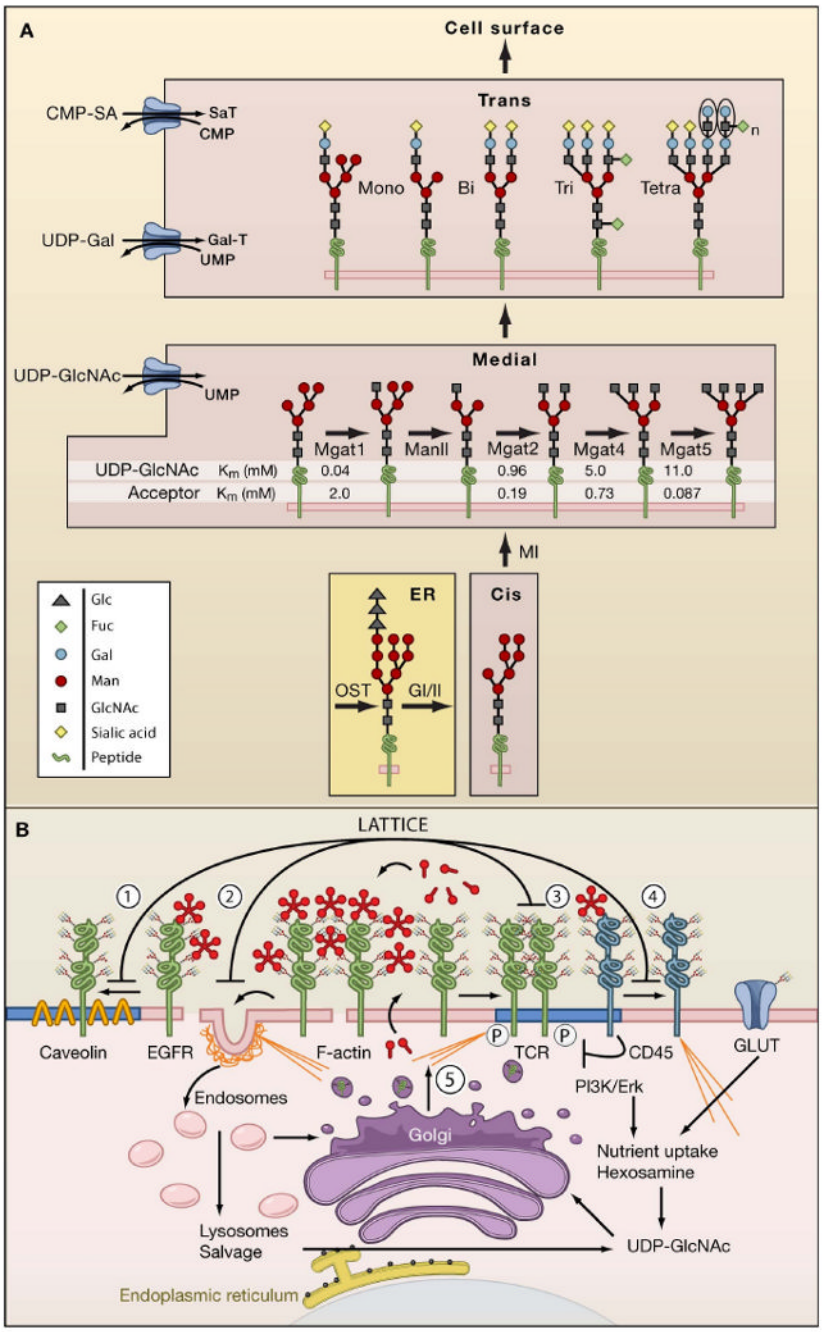

Figure 1. N-glycan branching pathways and the galectin lattice

(A) N-glycan branching pathway. Oligosaccharyltransterase (OT) utilizes the pre-assembled donor $Glc_3Man_9GlcNAc_2$-pp-dolichol to transfer the glycan to N-X-S/T motifs on glycoproteins in the endoplasmic reticulum (ER). Glycoproteins transit from the ER to cis, medial, and trans Golgi *en route* to the cell surface. The N-acetylglucosaminyltransferases enzymes, designated by their gene names (*Mgat1, Mgat2, Mgat4, Mgat5*) generate branched N-glycans that display a range of affinities for galectins. The K_m values for Mgat1, Mgat2, Mgat4 and Mgat5 are indicated as measured *in vitro* for UDP-GlcNAc and acceptor glycoproteins.

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(B) Dynamics of the galectin lattice. The glycocalyx is the thick carbohydrate layer surrounding the cell. Glycan structures generated in the Golgi differ in affinities for galectins. Galectins cross-link glycoprotein receptors and oppose (1) loss of epidermal growth factor receptors (EGFR) to Caveolin 1-positive microdomains, (2) coated-pit endocytosis, (3) precocious clustering of receptors, and (4) F-actin-mediated entry of T cell receptor (TCR) into and exit of CD45 from ganglioside GM1-positive microdomains (blue). (5) Nutrient supply and growth signaling increase membrane remodeling, regulate metabolite flux through the hexosamine pathway to UDP-GlcNAc and N-glycan branching (Golgi) on receptors to promote surface retention by the galectin lattice.

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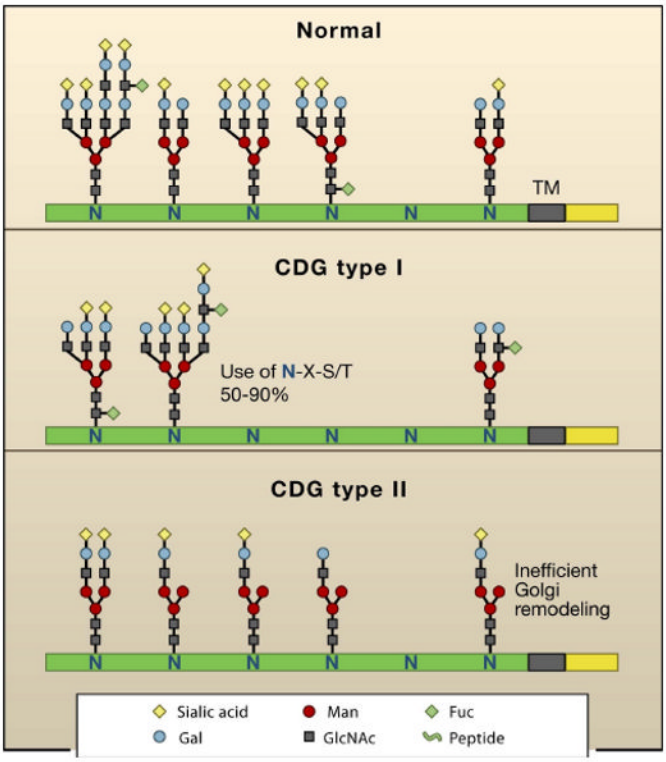

Figure 2. Congenital Disorders of Glycosylation and glycoforms

(A) Glycoprotein isoforms or glycofoms differ based on N-glycan structures present at each N-X-S/T site (not all sites are occupied). CDG type I alters glycoform distribution due to deficiencies in $Glc_3Man_9GlcNAc_2$-pp-dolichol biosynthesis leading to incomplete N-X-S/T site occupancy. CDG type II are deficiencies in Golgi remodeling or glycoprotein trafficking.

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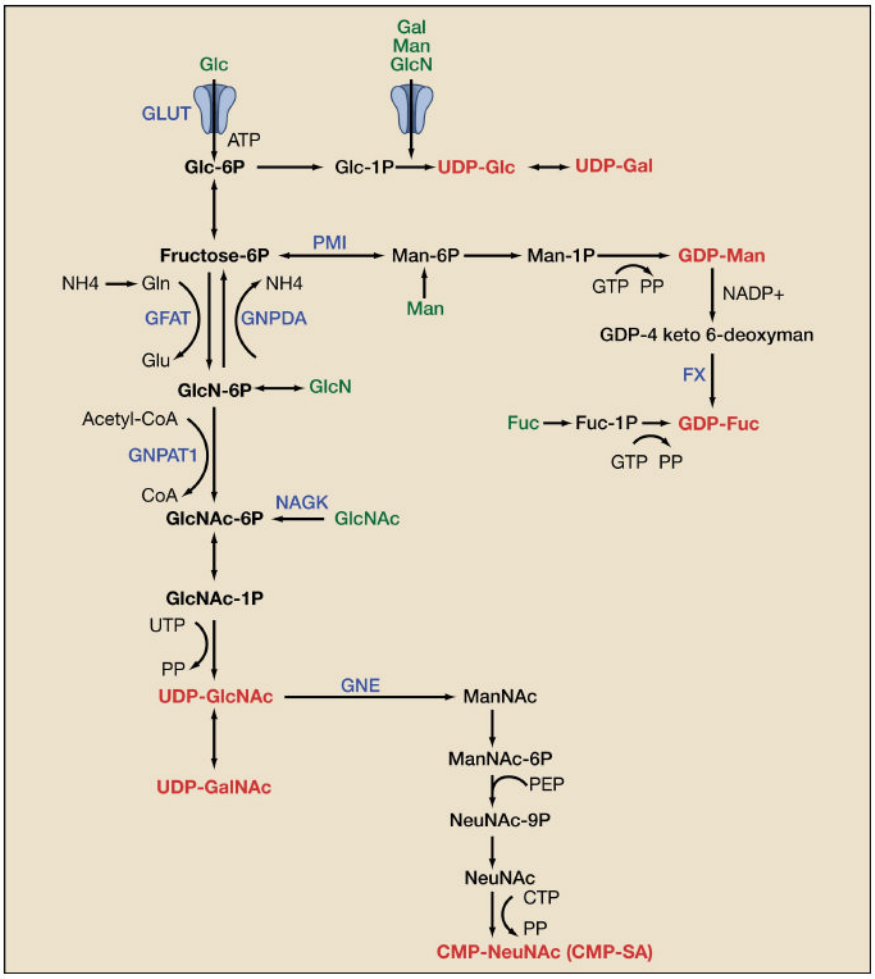

Figure 3. Biosynthesis of Sugar-nucleotides

De novo biosynthesis of UDP-GlcNAc is dependent on glucose, glutamine, acetyl-CoA and uridine. UDP-GlcNAc is also the precursor for UDP-GalNAc and CMP-SA biosynthesis. Gal, Man, and GlcN salvage/import (green) are facilitated by the hexose transporters (GLUTs), whereas GlcNAc and Fuc are taken up by bulk endocytosis. The sugar nucleotides are in red. Genes in blue are discussed in the text. Metabolite abbreviations: glucose (Glc), galactose (Gal), mannose (Man), glucosamine (GlcN), fucose (Fuc), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (NeuNAc), glutamine (Gln).

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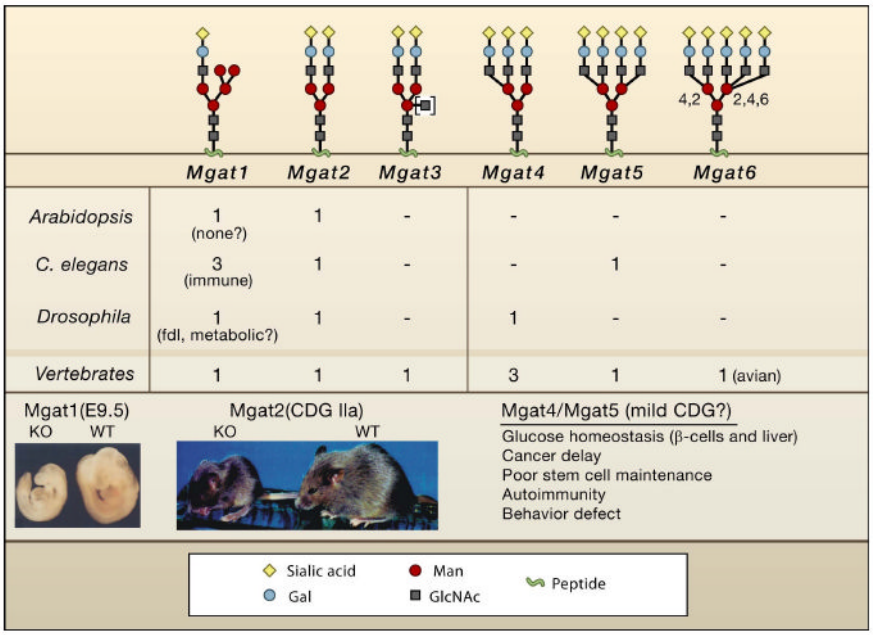

Figure 4. Mgat deficiencies

Listed are the number of genes encoding Mgat activities corresponding to the branching pattern shown above. *C. elegans* triple *Mgat1* mutant worms develop normally, but display altered sensitivities to pathogenic bacteria (Shi et al., 2006). Mutation of Mgat1 in *Drosophila* leads to fused lobes in the brain, and loss of motility in adult flies, possibly reflecting both developmental and metabolic deficiencies (Sarkar et al., 2006). A fused lobes (fdl) phenotype is also observed for loss of β-hexosaminidase, the enzyme that specifically removes GlcNAc added by Mgat1 (Leonard et al., 2006). It is possible that a precise level of Mgat1 product regulates a morphogen gradient that defines the boundary between lobes. Mgat1 deficient embryos are growth impaired and display defects in neural tube closure (Ioffe and Stanley, 1994; Metzler et al., 1994). Mice lacking Mgat2 are small and display severe defects in multiple organs, which is comparable to human CDG-IIa. Reproduced by permission of Oxford University Press: Wang et al. (2001) Glycobiology, 11 (12), 1051-1070. The product of Mgat3 shown in brackets can be found in any of the structures shown, but its presence blocks further branching.