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REVIEW

N-Glycans in cancer progression

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***N*-Glycan branching in the *medial*-Golgi generates ligands for lattice-forming lectins (e.g., galectins) that regulate surface levels of glycoproteins including epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) receptors. Moreover, functional classes of glycoproteins differ in *N*-glycan multiplicities (number of *N*-glycans/peptide), a genetically encoded feature of glycoproteins that interacts with metabolic flux (UDP-GlcNAc) and *N*-glycan branching to differentially regulate surface levels. Oncogenesis increases β1,6-*N*-acetylglucosaminyltransferase V (encoded by Mgat5) expression, and its high-affinity galectin ligands promote surface retention of growth receptors with a reduced dependence on UDP-GlcNAc. Mgat5$^{-/-}$ tumor cells are less metastatic in vivo and less responsive to cytokines in vitro, but undergo secondary changes that support tumor cell proliferation. These include loss of Caveolin-1, a negative regulator of EGF signaling, and increased reactive oxygen species, an inhibitor of phosphotyrosine phosphatases. These studies suggest a systems approach to cancer treatment where the surface distribution of receptors is targeted through metabolism and *N*-glycan branching to induce growth arrest.**

Keywords: cancer/cytokine signaling/metabolism/*N*-glycans

Cancer progression

Cancer invasion and metastasis is associated with changes in cell growth control and morphology. For example, expression of epidermal growth factor receptor (EGFR) family members in breast cancer correlates with aggressive tumor behavior and reduced survival time (Slamon et al. 1987). Treatment of patients with antireceptor antibodies and chemical inhibitors of these receptor tyrosine kinases (RTK) shows transient success at blocking tumor growth, but compensating up-regulation of other RTKs and signaling proteins can give rise to drug resistance (Baselga 2006). Activating mutations in genes that encode intracellular signaling proteins generally relax the requirements for extracellular growth factors in cancer cells. However, tumor cells remain dependent on growth factor and substratum adhesion to stimulate motility and invasion, which is characterized by epithelial-to-mesenchymal transition (EMT), loss of cell–cell adhesion junctions, and reorganization of actin microfilaments (Oft et al. 2002; Thiery 2003). Nonetheless, EGFR in endosomes can activate growth signaling with minimal occupancy by ligand (Reynolds et al. 2003; Offterdinger et al. 2004), and it has been suggested that growth signaling is largely constitutive were it not for extracelluar cues and receptors that suppress growth (Harris 2004). Here, we explore lectin–glycan interactions controlling the distribution of receptors at the cell surface, a poorly understood area, yet critical to a better understanding of cancer growth and metastasis.

Cytokine (growth factor) receptors are generally *N*-glycosylated transmembrane proteins, and residency at the surface is dependent in part on the dynamics of membrane remodeling. Endogenous lectins, such as galectins, can cross-link glycoproteins at the cell surface forming lattices (Lee RT and Lee YC 2000; Brewer et al. 2002) that enhance residency time at the cell surface (Demetriou et al. 2001; Lau et al. 2007) (Figure 1A). Lectin–glycoprotein lattices are transient (Pace et al. 1999), similar in this regard to focal adhesions and lipid raft microdomains which induce "molecular crowding" of integral membrane receptors required for complex formation and allosteric regulation of signaling (Kuriyan and Eisenberg 2007).

Galectins are *N*-acetyllactosamine (Galβ1-4GlcNAc)-binding proteins, expressed widely in metazoan tissues (Cooper 2002). Galectin polypeptides have either one or two carbohydrate reactive domains and form complexes that cross-link glycoproteins dependent on their glycan structures and concentrations. For example, the C-terminus of galectin-3 can form up to pentamers in the presence of multivalent ligands, generating a "lattice" microdomain with an irregular geometry (Ahmad et al. 2003). Galectins are produced in the cytosol and secreted by a nonclassical pathway where they first encounter glycoprotein ligands in cargo vesicles (Delacour et al. 2007) and at the cell surface (Hughes 1999). *N*-Glycans are the major ligands for galectin-1 and 3 at cell surfaces (Patnaik et al. 2006), but also bind to mucins and proteoglycans in extracellular matrix (Kinlough et al. 2004; He and Baum 2006). Galectin-3 has been shown to bind the *N*-glycans of EGFR, limiting its mobility in the plane of the membrane as measured by fluorescence recovery after photobleaching (Partridge et al. 2004; Lajoie et al. 2007). Oncogenic activation of the PI3K/Akt pathway enhances membrane remodeling and endocytosis, establishing conditions where glycoproteins become more dependent on the galectin lattice for surface retention (Partridge et al. 2004) (Figure 1A). However, oncogenesis also increases lattice avidity by up-regulating galectin-3 gene expression and higher affinity *N*-glycan ligands (Fernandes et al. 1991;

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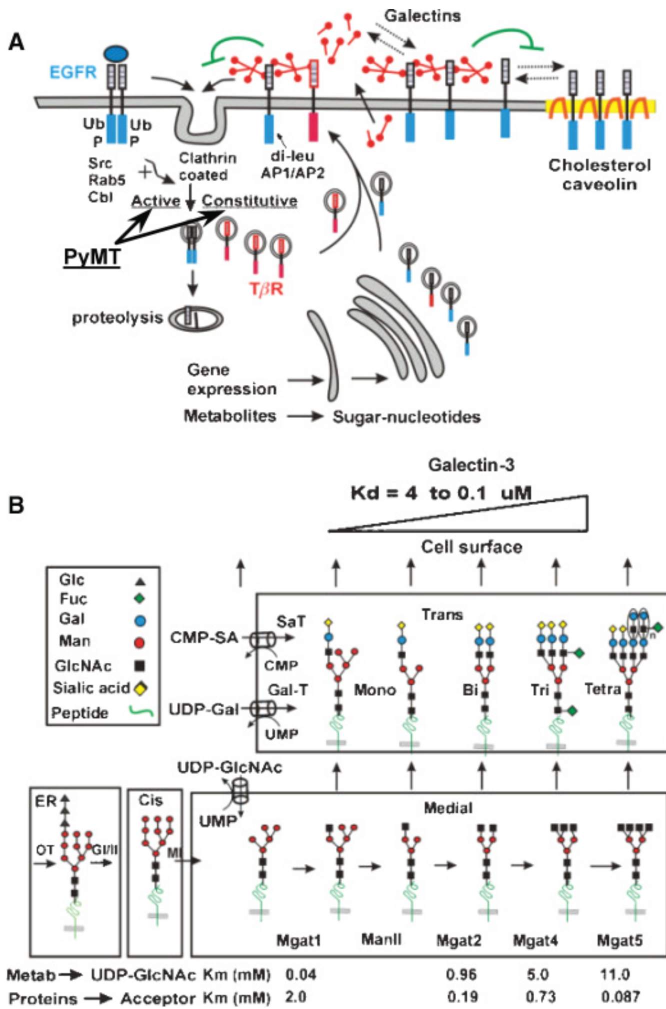

Fig. 1. (**A**) Model of lattice-dependent regulation of surface glycoproteins. Receptors are exchanged between the galectin lattice, Cav1-positive domains, and clathrin coated-pits. TβR is recycled constitutively, independent of ligand binding. In contrast, EGFR activation markedly enhances adaptor binding and endocytosis, while constitutive endocytosis of EGFR is relatively slow compared to TβR. The lattice preserves cytokine receptor sensitivity to ligands, and TβR is particularly sensitive to accelerated remodeling stimulated by transformation (PyMT oncoprotein). (**B**) 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, designated by their gene names (Mgat1, Mgat2, Mgat4, and Mgat5), generate branched *N*-glycans that display a range of affinities for galectins. The Mgat1, Mgat2, Mgat4, and Mgat5 Km values for UDP-GlcNAc and acceptors are indicated (sources listed in Lau et al. 2007).

Takenaka et al. 2004), presumably enhancing the availability of surface EGFR in a ligand-sensitive state. An EGFR mutation in human glioblastomas deletes a portion of the extracellular domain (EGFRvIII) leaving only 8 of 12 *N*-glycan sites and promotes self-association (Tsuda et al. 2000; Fernandes et al. 2001). The loss of *N*-glycans may reduce binding to galectin, resulting in precocious receptor dimerization and signaling.

Although galectins are the focus below, the siglecs also restrict the mobility of glycoproteins at the surface (Crocker et al. 2007). For example, siglec CD22 binds sialic acid (SA)-linked α2,6 to galactose and regulates B-cell receptor signaling (Collins et al. 2006). Terminal SA in the α2,6 but not α2,3 linkage reduces *N*-glycan affinities for galectin-1 (Toscano et al. 2007), suggesting the possibility that sialylation as well as *N*-glycan branching may interact to differentially regulate galectin- and siglecs-mediated lattices.

Mgat5 and signaling in tumor cells

Activating mutations in RAS, HER-2/NEU, and other oncogenes that result in gain-of-function in PI3K signaling are common events in human tumors (Fruman et al. 1998). As a model to study these events, polyomavirus middle T (PyMT) transgenic mice under the control of the MMTV promoter develop multifocal and metastatic mammary tumors (Guy et al. 1998). The PyMT oncoprotein is an inner-membrane scaffold protein that recruits Shc/Ras, Src, and p85/PI3K/Akt. Carcinomas in PyMT mice display a longer latency and fewer metastases on a $Mgat5^{-/-}$ background, indicating a role for *N*-glycan branching in tumor progression (Granovsky et al. 2000). β1,6-*N*-acetylglucosaminyltransferase V (gene and enzyme are referred to here as Mgat5) adds GlcNAc in a β1,6-linkage, initiating the fourth branch in a sequential pathway to tetraantennary *N*-glycans (Figure 1B). The affinity of *N*-glycans for these galectins increases in proportion to branching presumably due to rebinding and slower off-rates (Dam et al. 2005). The Mgat5 product is the preferred intermediate for extension with poly-*N*-acetyllactosamine, which also enhances affinity for galectin-1, -3, -8, and -9 (Hirabayashi et al. 2002).

N-Glycosylation begins with cotranslational transfer of $Glc_3Man_9GlcNAc_2$ by oligosaccharyltransferase to ∼70% of lumenal N-X-S/T (where X $\neq$ Pro) motifs (Apweiler et al. 1999). After the transfer and trimming of two glucose (Glc) units, the protein chaperones calnexin and calreticulin bind to $Glc_1Man_9GlcNAc_2$ and the remaining Glc residue is cycled by α-glucosidase II and ER α-glucosyltransferase until protein folding is complete (Helenius and Aebi 2004). Chaperone-assisted protein folding employs an ancient and relatively homogenous structure in the *N*-glycan pathway prior to the Golgi (Banerjee et al. 2007). However, evolution of the metazoan Golgi pathways has given way to an explosion in *N*-glycans structural diversity. Mannose residues are removed in the *cis*-Golgi, and the *N*-acetylglucosaminyltransferases I, II, IV, and V, (encoded by Mgat1, Mgat2, Mgat4a/b, Mgat5) initiate the branches in a sequential and ordered manner, but with decreasing efficiency (Schachter 1986) (Figure 1B). β4-galactosyltransferases in the *trans*-Golgi substitute GlcNAc branches with little selectivity to generate *N*-acetyllactosamine (Togayachi et al. 2006), the epitope for galectin binding. Mgat3 adds *N*-acetylglucosamine (GlcNAc) to the inner β-linked mannose, which is not elongated and blocks further branching (Yoshimura et al. 1995).

Akt-p(473) levels are reduced in early-stage mammary tumors from PyMT $Mgat5^{-/-}$ mice indicating that β1,6GlcAc-branching plays a role in oncogenesis (Granovsky et al. 2000). We also observed that PyMT $Mgat5^{-/-}$ tumor cells were generally less sensitive to acute stimulation by EGF, IGF, PDGF, bFGF, and TGF-β (Partridge et al. 2004). Surface EGFR and transforming growth factor-β receptors (TβR) were reduced while their residency in the endosomes was increased. Further analysis indicated that galectin binding opposes the loss of receptors to endocytosis, and indeed, when chemical inhibitors of endocytosis were used, sensitivity to EGF and TGF-β was

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rescued in PyMT Mgat5$^{-/-}$ tumor cells. Inhibiting endocytosis did not enhance the already robust response to cytokines in PyMT Mgat5$^{+/+}$ cells, although disrupting the galectin lattice did reduce surface receptor residency. PyMT Mgat5$^{-/-}$ tumor cells maintained an epithelial morphology with cell–cell adhesion junctions containing E-cadherin. In contrast, PyMT Mgat5$^{+/+}$ tumor cells displayed a mesenchymal appearance with loss of E-cadherin (Partridge et al. 2004). Importantly, expression of Mgat5 from a retroviral vector in PyMT Mgat5$^{-/-}$ cells induces loss of E-cadherin, cell motility, restored sensitivity to cytokines, and enhanced the metastatic phenotype when the cells were injected into mice. The Mgat5 (L188R) mutation, which blocks enzyme localization to Golgi (Chaney et al. 1989), failed to rescue signaling and EMT indicating a requirement for the *N*-glycan product of the enzyme (Partridge et al. 2004).

Mgat5 interacts with caveolin-1

Although mammary tumors in PyMT Mgat5$^{-/-}$ mice grew more slowly than tumors in PyMT Mgat5$^{+/+}$ mice, a minority displayed a late-phase increase in the growth rate (Granovsky et al. 2000). Activated Akt-p(473) levels were increased in these "escapers" tumors, reaching levels comparable to that of PyMT Mgat5$^{+/+}$ tumors, indicating a recovery of PI3K/Akt signaling (Figure 2). The escaper tumors display a significant reduction in caveolin-1 (Cav1) expression, and surprisingly, near normal sensitivity to EGF. However, TGF-β sensitivity remained low and the cells failed to undergo EMT (Lajoie et al. 2007). Cav1, a nonglycosylated integral membrane protein, binds cholesterol in lipid rafts and is found in cavoelae microdomains that can mediate noncoated pit endocytosis (Pelkmans et al. 2005). Signaling proteins including Src kinases and H-Ras are enriched in lipid rafts where their clustering can enhance activation. Cav1 has a "scaffolding domain" (residues 82–101) that binds a conserved motif found in many signaling proteins and suppresses their activity (Okamoto et al. 1998). Loss of Cav1 in PyMT Mgat5$^{-/-}$ cells appears to compensate for decreased levels of surface EGFR (Figure 1A). Restoring Cav1 expression in the escaper PyMT Mgat5$^{-/-}$ cells suppressed EGFR signaling as might be expected, but Cav1 overexpression had no effect in PyMT Mgat5$^{+/+}$ tumor cells, suggesting that the Mgat5-fortified lattice acts dominantly (Lajoie et al. 2007). The CAV1 gene maps to a tumor suppressor locus (D7S522; 7q31.1) and is frequently deleted or mutated in human carcinomas (Lee, Park, et al. 2002), possibly an event that precedes up-regulation of Mgat5 gene expression.

Interestingly, caveolin/cholesterol is required for Ras signaling early in *Caenorhabditis elegans* development (Scheel et al. 1999). *C. elegans* and vertebrates produce cholesterol, and their genomes encode caveolin, Mgat5, and ~15 galectin homologs. *Drosophila* lack caveolin, Mgat5, produce the evolutionary precursor of cholesterol (ergosterol) and have only a single monovalent galectin. Cholesterol and ergosterol impart different dynamics to the membrane (Shrivastava and Chattopadhyay 2007), and therefore it is possible that caveolin/cholesterol-rafts and galectin/*N*-glycan-lattice coevolved as part of a larger network for glycoprotein regulation by microdomains.

Mgat5 and Pten interact

Pten is the phosphoinositide 3-phosphatase that opposes growth receptor signaling via PI3K/Akt by reducing phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) levels (Cantley 2002) (Figure 2). Pten haploinsufficiency causes multifocal cancers, loss of adult stem cells in mice, and alterations in the pathway occur frequently in human malignancies (Inoki et al. 2005). Since PI3K signaling is reduced in early-stage PyMT Mgat5$^{-/-}$ tumors, we tested whether the Mgat5 deficiency could also reduce growth signaling in Pten$^{+/-}$ mice. Survival of Mgat5$^{-/-}$ mice is reduced to ~80% with an apparent early aging phenotype, while Pten$^{+/-}$ lifespan is <40% of the wild-type mice due to early lymphoma and other tumors. However, compound Mgat5$^{-/-}$/Pten$^{+/-}$ mutant mice display an intermediate longevity, suggesting that the Mgat5 mutation delays the onset of Pten$^{+/-}$ lymphomas (Cheung and Dennis 2007).

To explore the interaction between Mgat5 and Pten in premalignant cells, we examined phenotypes in mouse embryonic fibroblasts. Mgat5$^{-/-}$ and Pten$^{+/-}$ double-mutant cells display lower Akt-p(473), cell spreading, and growth rates compared to Pten$^{+/-}$ alone, indicating that the Mgat5-deficiency suppressed some aspects of the Pten$^{+/-}$ phenotype. Pten$^{+/-}$ fibroblasts also showed enhanced β1,6GlcNAc-branching in the Mgat5$^{+/-}$ and Mgat5$^{+/+}$ backgrounds, consistent with reports that H-Ras activates Mgat5 gene expression (Kang et al. 1996; Buckhaults et al. 1997). However, microfilament morphology and polarity of the cells were not completely normalized in double mutant cells, suggesting that a diploid dosage of both Mgat5 and Pten is needed for optimal microfilament dynamics and membrane remodeling (Cheung and Dennis 2007). Pten at the trailing end of motile cells creates regional concentration gradients of PtdIns(3,4,5)P_3 (Funamoto et al. 2002; Vazquez et al. 2006) that polarizes the cytoskeleton in filopodia (Weiner et al. 2002). We conclude that lattice avidity and PI3K signaling work in opposition to promote intracellular signaling gradients required for cell motility. The ratio of galectin-3 to glycoprotein ligands also displays an optimum for PI3K/Atk signaling, microfilament turnover, and cell motility (Lagana et al. 2006).

Reactive oxygen species (ROS) and signaling

Cellular ROS is largely a by-product of oxidative respiration that damages many cellular constituents, notably causing mutations and inhibiting gene promoters with aging (Lu et al. 2004). However, ROS (i.e., H_2O_2) is also a necessary mediator of signaling in eukaryotes (Brunet et al. 2004). H_2O_2 reversibly titrates a redox-sensitive and essential thiol group found in the active site of phosphotyrosine phosphatases (PTPs) (e.g., PTEN, LAR, PTP1, LMW-PTP) resulting in enhanced basal tyrosine kinase signaling (Lee, Yang, et al. 2002; Nimnual et al. 2003; den Hertog et al. 2005). H_2O_2 is also generated directly as a signaling intermediate by NADPH-dependent oxidase (NOX) upon Rac1 recruitment to activated PDGF and EGF receptors (Nimnual et al. 2003) (Park et al. 2004). This H_2O_2 inhibits the low-molecular-weight protein tyrosine phosphatase (LMW-PTP), thereby increasing tyrosine phosphorylation and activation of p190Rho-GAP. Rac and Rho GTPases have opposing activities that regulate microfilament remodeling. Galectins are also redox sensitive (Hirabayashi and Kasai 1991), and ROS may also inhibit lattice avidity thereby influencing receptor residency.

PTPs maintain receptors in an active state, thus sensitizing the cell to extracellular ligands. However, oxidation of PTPs in the endomembranes can sustain EGFR activation in a largely ligand-independent manner (Reynolds et al. 2003; Offterdinger et al.

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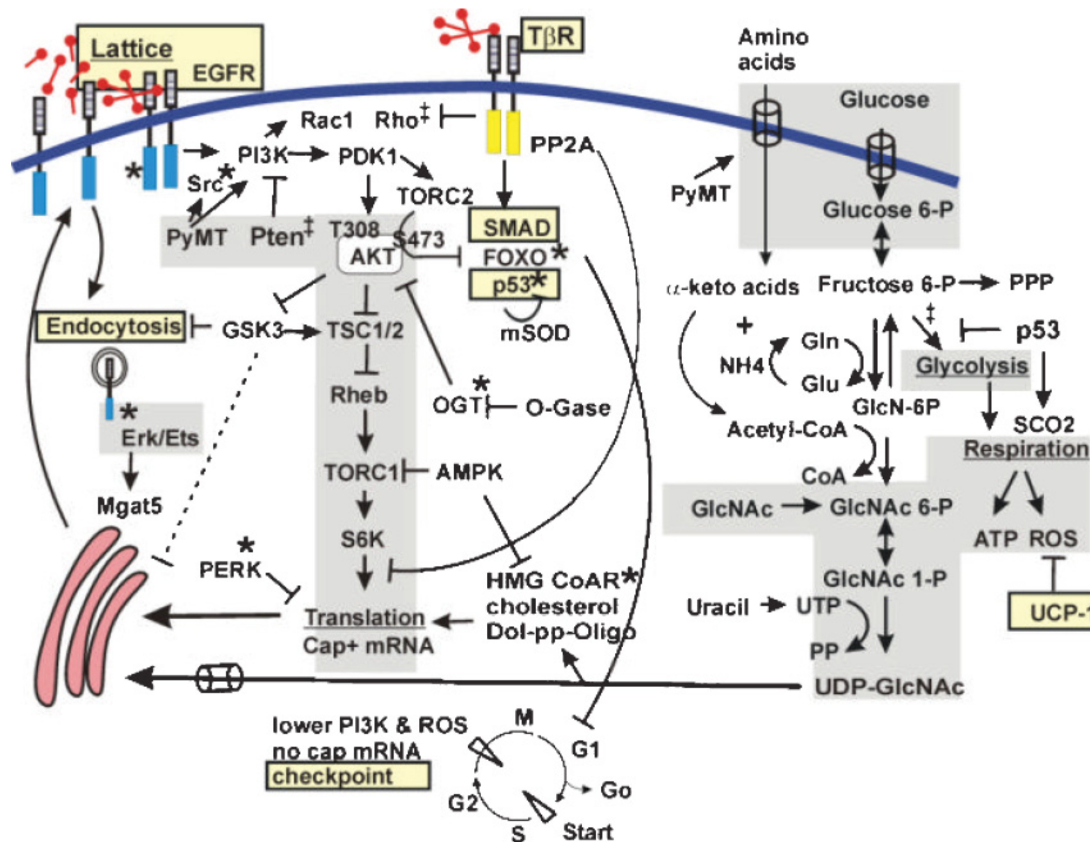

Fig. 2. Signaling, metabolism, glycoprotein biosynthesis, and cell cycle. The hexosamine pathway and UDP-GlcNAc supply to the Golgi are sensitive to glucose (Glc), glutamine (Gln), acetyl-CoA, glucosamine (GlcN), and *N*-acetylglucosamine (GlcNAc). As detailed in the test, *indicates activation and ‡inhibition by ROS (H_2O_2). The gray and yellow colors indicate enhanced and reduced activities, respectively, in PyMT Mgat5$^{-/-}$ tumor cells compared to PyMT Mgat5$^{+/+}$ counterpart. The dashed line is inferred from GSK3 inhibition and presently under investigation.

2004). ROS generated by the electron transport chain is likely to be persistent, whereas H_2O_2 production via Rac-1/NOX is local and transient (Reynolds et al. 2003). In Mgat5$^{-/-}$ tumor cells, the fraction of RTKs in the endosomes is increased (Partridge et al. 2004). Moreover, Mgat5$^{-/-}$ cells show increased glucose uptake, ROS, protein synthesis, and Akt-p/Erk-p, and unlike wild-type cells, they fail to down-regulate upon serum-withdrawal. Elevated ROS signaling in Mgat5$^{-/-}$ tumor cells is dependent on glucose metabolism, as uncoupling of oxidative respiration or the TORC1 inhibitor rapamycin normalized signaling. A similar relationship between ROS and TORC1 has been observed in BCR-ABL-transformed cells (Kim et al. 2005). Although internal generation of ROS can stimulate growth signaling, Mgat5 is necessary for responsiveness to negative extracellular cues that restrain proliferation under low serum conditions (Mendelsohn et al. 2007). This surprising result suggests that Mgat5 and the lattice play a role in arrest as well as growth stimulation.

PyMT Mgat5$^{-/-}$ cells fail to arrest when treated with the M-phase blocker colcemid or the S-phase blocker hydroxyurea indicating a checkpoint deficiency. Cells transit the cell cycle with shorter G1 and G2 gap phases (Mendelsohn et al. 2007). PI3K/Akt and Cdc2 kinase activities increase during S-phase and are required for mitotic entry (Roberts et al. 2002). It is possible that a persistence of high ROS in PyMT Mgat5$^{-/-}$ cells blocks down-regulation of Akt in G2 and speeds re-entry into S-phase. This is consistent with the smaller size of PyMT Mgat5$^{-/-}$ cells and higher fraction of the cells in S-phase. However, oxidative stress also stimulates the acetylation of FOXO and p53, increasing the expression of checkpoint and antioxidant proteins (Brunet et al. 2004; Essers et al. 2005) (Figure 2). Nonetheless, damage-induced S- and M-phase checkpoints are deficient in PyMT Mgat5$^{-/-}$ tumor cells, while rates of replication and death are increased (Mendelsohn et al. 2007). These results suggest that Mgat5 expression in tumor cells regulates cell-cycle progression, presumably by sensitizing cells to extracellular cues for arrest signaling. The weaker lattice in PyMT Mgat5$^{-/-}$ cells is insufficient to maintain surface levels of low-multiplicity glycoproteins such as TβR. One possibility is that reduced TGF-β-dependent activation of Smad2/3 and complex formation with FOXO and p53 contributes to the up-regulation of c-Myc and cell-cycle progression (Cordenonsi et al. 2003; Seoane et al. 2004). Importantly, these results suggest that cell-cycle progression is dependent on Mgat5/*N*-glycan branching, presumably in their capacity to balance the proportions of surface receptors (Mendelsohn et al. 2007).

Hexosamine rescue of the lattice

GlcNAc supplementation to PyMT Mgat5$^{-/-}$ tumor cells increased UDP-GlcNAc by 3- to 4-fold and triantennary *N*-glycans by 2-fold (tetra-antennary are not restored) (Lau et al. 2007). GlcNAc increased galectin-dependent binding to EGFR

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and TβR in PyMT Mgat5$^{-/-}$ tumor cells, rescuing surface expression, sensitivity to cytokines, and EMT. GlcNAc also restored negative regulation of glucose uptake, ROS, and the cell cycle in PyMT Mgat5$^{-/-}$ cells (Mendelsohn et al. 2007). This suggests that *N*-glycans with different numbers of branches function in an additive and redundant manner to retain glycoproteins at the surface.

GlcNAc and GlcN are normally salvaged from glycoconjugate turnover by hydrolysis of glycosidic linkages in lysosomes and transport of the amino-sugars into the cytoplasm by an uncharacterized transporter, where they are phosphorylated and re-enter the UDP-GlcNAc pool (Figure 2). There are two routes in mammalian cells for GlcN-6P, either acetylation to GlcNAc-6P (GNPAT1/Emeg32) (Boehmelt et al. 2000) or deamination and conversion to fructose-6P by glucosamine-6-phosphate isomerase/oscillin (GNPDA) (Zhang et al. 2003). In contrast, the only route for GlcNAc-6-P, the product of GlcNAc-6-kinase (NAGK), is directly into UDP-GlcNAc. Salvage of GlcNAc into Golgi pathways is more direct than GlcN, as potent feedback inhibition of GFAT by GlcN-6P (Broschat et al. 2002), and GNPDA can send GlcN to fructose-6P and glycolysis. Thus, GlcNAc is spared from catabolism in mammalian cells, efficiently salvaged into UDP-GlcNAc and Golgi pathways.

Central metabolites in the cell including glucose, glutamine, acetoacetate, and uridine can be limiting for de novo synthesis of UDP-GlcNAc (Grigorian et al. 2007). For example, T-cell activation induces large increases in glucose uptake (Frauwirth et al. 2002), but supplementation with hexosamine metabolites can further increase UDP-GlcNAc levels and *N*-glycan branching. Activated T cells proliferate rapidly, but ultimately arrest, and this requires CTLA-4 receptor binding to galectin and retention at the cell surface (Lau et al. 2007).

Galectin lattice regulation by N-glycan branching

GlcNAc-branching is an ordered sequential pathway, but structures at each N-X-S/T site occur with probabilities dependent on *N*-acetylglucosaminyltransferase activities, flux of substrate glycoproteins, and UDP-GlcNAc levels. Ordinary differential equations have been used to model the Golgi pathway using enzyme concentrations and kinetic parameters from the literature (Umana and Bailey 1997). Focusing on the high-affinity galectin ligands, the tri- and tetraantennary *N*-glycans increase in an ultrasensitive manner as a function of increasing UDP-GlcNAc (Lau et al. 2007). Ultrasensitivity describes a stimulus/response relationship where the response is delayed, then rises sharply over a narrow range of stimulus (a sigmoid curve) with a Hill coefficient (nH) $\gg 1$ indicating the deviation from a typical hyperbolic response curve (Koshland et al. 1982). Experimentally, GlcNAc titration into cultured cells increases UDP-GlcNAc concentrations with Michaelis–Menten kinetics, but the tri- and tetraantennary *N*-glycans increase with sigmoidal kinetics (Figure 3A). These dynamics are due to biochemical features of the pathway, notably decreasing enzyme activities moving down the branching pathway, decreasing affinities for a common substrate (UDP-GlcNAc), a strict sequential order of reactions, and limited reaction times due to transit of glycoprotein substrates through the Golgi (Lau et al. 2007).

The Golgi UDP-GlcNAc antiporter (SLC35A) exchanges UMP for UDP-GlcNAc and establishes a direct proportionality between the steady-state amounts of UDP-GlcNAc inside the Golgi and in the cytosol (Waldman and Rudnick 1990). However, the number of transporters and consumption of UDP-GlcNAc due to the rates of protein flux through the Golgi may also determine availability. The Golgi concentration of UDP-GlcNAc has not been measured directly, but estimates have been made of ~1.5 mM, representing ~15 times that of cytoplasmic (Waldman and Rudnick 1990). The first branching enzyme, Mgat1 binds UDP-GlcNAc efficiently, but affinity for the acceptor *N*-glycan is relatively low with Km 0.04 and ~2 mM, respectively (Figure 1B). This relationship is reversed for Mgat4 and Mgat5 enzymes suggesting that Mgat1 is dependent on glycoprotein acceptor concentrations and transit rates through the Golgi, while Mgat4 and Mgat5 are limited by UDP-GlcNAc concentrations (Lau et al. 2007). In other words, *N*-glycan branching capacity is suboptimal and limited by the rate of protein synthesis prior to Mgat2 action, and thereafter by the UDP-GlcNAc concentration. In addition, factors regulating α-mannosidase I and II activities can determine the access of Mgat2 to substrates, and Mgat3 activity blocks further branching (Schachter 1986). Thus, the influence of the galectin lattice on surface levels of glycoproteins is expected to be dependent on bulk protein synthesis rates, Golgi enzyme activities, and metabolite concentrations supporting UDP-GlcNAc biosynthesis.

Ultrasensitivity to UDP-GlcNAc provides an important clue to our understanding of *N*-glycan function. Remarkably, this pathway feature provides a mechanism for differential regulation of glycoproteins at the cell surface based on *N*-glycan number and metabolite flux to UDP-GlcNAc (Lau et al. 2007). More than 40 *N*-glycan structures (variations on branching and extension) have been identified on EGFR (Stroop et al. 2000). The protein environment of N-X-S/T sites can limit the accessibility to branching enzymes (Do et al. 1994), but for 8 occupied N-X-S/T sites in EGFR and a conservative estimate of 14 possible structures at each site, we have a theoretical 203,490 glycoforms, ~10 times the number of surface EGFR molecules per cell (DeWitt et al. 2002; Lau et al. 2007). The glycoform number increases exponentially with multiplicity that widens the range of affinities for galectins (Figure 3B). The steady-state distribution of glycoproteins at the cell surface can be calculated based on trafficking rates and binding avidities of the various glycoforms for galectin-3. Consistent with experimental data, a major fraction of the growth-promoting RTKs (e.g., EGF, INS, PDGF) with multiplicities >5 are retained by the lattice at the basal Golgi UDP-GlcNAc concentration (~1.5 mM), and increases occur in a graded manner downstream of increasing UDP-GlcNAc (1.5 to 6 mM). In contrast, glycoproteins with <5 *N*-glycans (e.g., TβR) are largely below the threshold for stable association, but increased branching can generate higher affinities and stable association with galectin at the cell surface. Also consistent with the experimental data, low-multiplicity glycoproteins increase with a sigmoidal response to UDP-GlcNAc, mirroring the ultrasensitive output of tri- and tetraantennary *N*-glycans (Figure 3C).

Evolution of multiplicity in receptor kinases

Hexosamine/*N*-glycan branching appeared to differentially regulate EGFR/PI3K/Erk and TGF-β/Smad signaling pathways that are antagonistic for G1/S transition (Matsuura et al. 2004). If more broadly, glycoproteins with opposing roles in growth and

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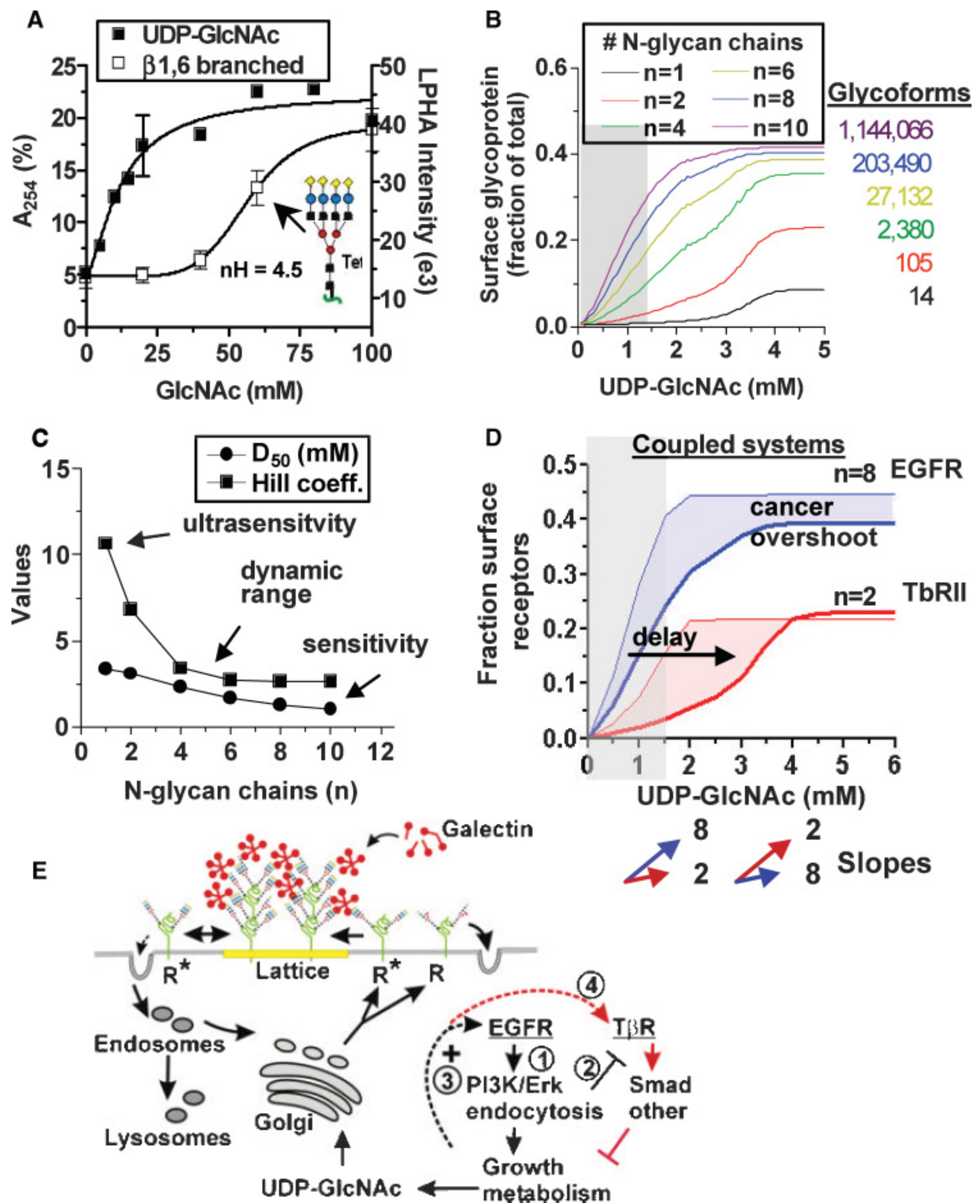

Fig. 3. Modeling the interaction between *N*-glycan multiplicity and Mgat expression. (**A**) UDP-GlcNAc and tetraantennary *N*-glycans levels increase in response to GlcNAc salvage displaying hyperbolic and ultrasensitive responses, respectively. NMuMG epithelial cells were cultured in a medium supplemented with GlcNAc for 48 h, branching was quantified by probing with FITC-L-PHA, and UDP-GlcNAc measured by HPLC. L-PHA lectin binds to β1,6GlcNAc-branched (Mgat5-dependent) *N*-glycans. (**B**) Fractional increases in surface glycoproteins as a function of Golgi UDP-GlcNAc and *N*-glycan multiplicities (*n*). (**C**) Ultrasensitivity (Hill coefficient), dynamic range (Vo-Vmax), and sensitivity (D_{50}) for responses to increasing UDP-GlcNAc as a function of multiplicity (*n*). (**D**) Change in surface levels of TβR ($n = 2$) and EGFR ($n = 8$) in Mgat5$^{-/-}$ (heavy lines) and Mgat5$^{+}/^{+}$ tumor cells (thin lines) with increasing UDP-GlcNAc, respectively. The blue and red shading represents up to 15-fold increase in Mgat4/Mgat5/iGNT (*simulating transformed cells*), beginning from a simulated nontransformed Mgat5$^{+}/^{+}$ baseline. The gray box represents UDP-GlcNAc values that are below the estimated physiological ~1.5 mM level. Cancer-associated changes result in insensitivity to UDP-GlcNAc and selective increases in higher multiplicity glycoproteins (EGFR). (**E**) Model for growth (EGFR) and arrest (TβR) signaling based on difference in *N*-glycan multiplicity, UDP-GlcNAc supply to branching, and association with the galectin lattice. Glycoforms generated in the Golgi are above (R*) or below (R) the affinity threshold for stable association with the galectin lattice. With insufficient positive feedback to growth signaling (black) results in a predominance of arrest signaling (red). (1) stimulation of RTKs in quiescent cells (2) increases PI3K signaling promotes membrane remodeling and preferential internalization of low n receptors. (3) Positive feedback to hexosamine/*N*-glycan processing (Mgat4/Mgat5 expression), leads ultimately to (4) increasing TβR affinity for galectins and autocrine arrest signaling. (From Lau et al. 2007)

arrest are coupled by metabolic regulation of their *N*-glycans, we reasoned that this might be reflected in the *N*-glycan multiplicity of different functional classes of receptors. To explore this possibility in a relatively well-studied set of human receptors, we ranked the receptor kinases by the incidence of canonical *N*-glycosylation motifs (N-X-S/T not followed by P, X not P) in their extracellular domains. Mammalian receptor kinases that stimulate cell proliferation, growth, and oncogenesis have more N-X-S/T sites, longer extracellular domains, and an increased number of sites per 100 amino acids, with a mean of 11.33 ± 5.05 sites/receptor and density of 1.89 ± 0.58 sites/100 amino acids (Lau et al. 2007). These receptor kinases stimulate growth

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and proliferation (INSR, EGFR, PDGFR, etc.) and have on average ∼5 times more N-X-S/T sites, longer extracellular domains, and more sites per 100 amino acids compared to receptor kinases that mediate organogenesis and terminal differentiation (Tie1, Musk, Ltk, ROR1/2, DDR1, TβR, and EphR). These observations suggest that differences in *N*-glycan multiplicity between functional classes of receptor kinases are conserved. More importantly, *N*-glycan multiplicity (*n*), a gene sequence-encoded feature of glycoproteins allows differential regulation of surface receptors by nutrient flux to UDP-GlcNAc and the Golgi *N*-glycan pathways.

Coupled pathways regulated via hexosamine/Golgi/lattice

In nontransformed epithelial cells, hexosamine/Golgi/lattice has properties of a self-limiting or oscillating regulatory circuit (Lau et al. 2007). The same stimulus (UDP-GlcNAc) promotes a Michaelis–Menten and a sigmoidal increase in EGFR and TβR levels, respectively, with an intervening delay (Figure 3D). This delay between response curves for low- and high-multiplicity glycoproteins represents a window for increasing UDP-GlcNAc where growth signaling can increase in intensity (e.g., EGFR) before the onset of negative regulation by "coupled" low-multiplicity receptor systems (e.g., TβR) (Figure 3D and E). Growth factor receptors (e.g., IGFR, EGFR, PDGF, etc.) and morphogen receptor kinases (e.g., TβR, EphR, etc.) interact at multiple levels, but ultimately cell-cycle arrest is required for tissue homeostasis. TGF-β acts as an inhibitor of epithelial cell growth and slows cancer initiation, but once this barrier is overcome, TGF-β signaling enhances invasion (Cui et al. 1996). This apparent biphasic action of TGF-β on cell phenotypes is reflected at multiple levels of signaling. TGF-β stimulates TβRII/PAR6/PKCγ/SMURF1 which regulates the antagonistic activities of RhoA and Rac1 in microfilament remodeling (Ozdamar et al. 2005). TGF-β enhances collagen, fibronectin, and proteoglycans expression, which can stimulate focal adhesion signaling, but is inhibitory at high density where cell-substratum contacts are high avidity (Palecek et al. 1997). Canonical TβR/SMAD2/3 signaling slows cell-cycle progression by suppressing c-myc expression (Matsuura et al. 2004; Seoane et al. 2004) and recruiting protein phosphatase 2A (PP2A) which inhibits TORC1/S6K and protein synthesis (Petritsch et al. 2000) (Figure 2).

TGF-β/BMP signaling is required for terminal differentiation in most tissues during embryogenesis. In cultures of human embryonic stem cells, avoiding terminal differentiation in favor of self-renewal requires precise proportions of fibroblast growth factor (bFGF) and Noggin, a bone morphogenic protein (BMP)/TGF-β antagonist (Xu et al. 2005). In cancer, exogenous BMP4 shifts the equilibrium toward differentiation in tumor cells and can induce growth–arrest of human glioblastoma tumors in nude mice (Piccirillo et al. 2006).

The premalignant cell lines NMuMG and HEK293 epithelial cells are sensitive to increasing GlcNAc supplementation: first showing RTK/Erk-p then TβR/Smad2/3-p signaling (Lau et al. 2007). However, PyMT Mgat5$^{+/+}$ tumor cells appear to be insensitive to GlcNAc, as tri- and tetraantennary *N*-glycans as well as signaling increase only slightly with supplementation. Oncogenic activation of PI3K and Erk/Ets increases Mgat4, Mgat5, and β1,3*N*-acetylglucosaminyltransferase (iGNT) expression and their *N*-glycan products, which are the high-affinity galectin ligands (Kang et al. 1996; Buckhaults et al. 1997; Takamatsu et al. 1999; Ishida et al. 2005). Increased levels of Mgat4 and Mgat5 enzymes compensate for their lower UDP-GlcNAc affinities, essentially reducing the UDP-GlcNAc requirement (apparent D_{50}) of the branching pathway to tetraantennary *N*-glycans. In human carcinomas of breast and colon, increased β1,6GlcNAc-branching correlates with decreased survival time (Seelentag et al. 1998). Modeling the effects of increased expression of these enzymes not only confirmed this shift in D_{50} but also revealed a preferential increase in high-multiplicity receptors at normal physiological levels of UDP-GlcNAc (∼1.5 mM) (Figure 3D) (Lau et al. 2007). Thus, transformation promotes surface residency of growth receptors while "uncoupling" the hexosamine/Golgi/lattice network for sensitivity to TGF-β and other factors that restrain growth (Figure 4).

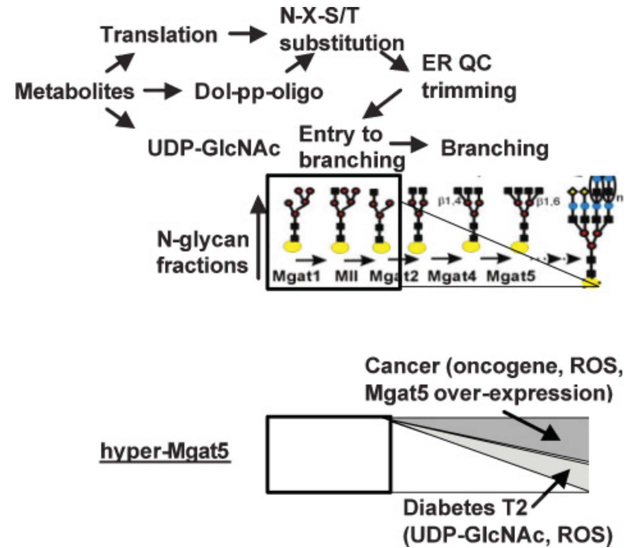

Fig. 4. Schematic of inputs to *N*-glycan branching triangles represent the proportions of branched *N*-glycans bi-, tri-, and tetra antennary *N*-glycans generated by the Golgi pathway. Oncogene-driven positive feedback to *N*-glycan branching in cancer, and altered metabolism associated with insulin resistance may enforce mal-adaptation of signaling at the cell surface.

Other pathways requiring UDP-GlcNAc

Glycopolymers other than *N*-glycans may contribute to cellular phenotypes downstream of GlcNAc supplementation. A global picture of the up- and down-regulated genes indicates that supplementation with high GlcNAc reduces gene expression downstream of EGFR and MAPK pathway, while G1/S checkpoint genes are increased (Lau et al. 2008). Interestingly, fatty acid biosynthesis transcripts are increased in low GlcNAc and decreased in high GlcNAc. Metabolic pathways were generally decreased in high GlcNAc, correlating in this regard with growth–arrest. A subset of genes that showed increased expression in high GlcNAc were suppressed using small interfering RNA (siRNA) and assessed for reversal of the high GlcNAc-induced phenotype; notably reduced proliferation, endocytosis, and increased β1,6GlcNAc-branching. siRNA targeting MGAT5, MAN2A2, LGALS3, WBSCR17, PHF3, SDC2, and CTNNAL1 partially reversed the three GlcNAc-induced phenotypes, suggesting an interaction between galectin-3/*N*-glycans, proteoglycans, *O*-glycans, and junctional cell

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adhesion. As high GlcNAc supplementation induces an EMT-like phenotype in NMuMG epithelial cells, these siRNA are candidate suppressors of metastatic activity (Lau et al. 2007).

Upstream of N-glycan branching

Factors affecting multiplicity include $Glc_3Man_9GlcNAc_2$-pp-dol levels and the efficiency of *N*-X-S/T substitution in the ER, chaperone-assisted protein folding and trimming by α-glucosidases and α-mannosidases prior to GlcNAc-branching (Figure 4). Golgi mannosidase and Mgat1 activities are generally not saturating, suggesting that the mass of glycoproteins trafficking through the Golgi is a factor in glycoform distribution. We might expect that the flux of glycoproteins through the Golgi to be coupled with flux of metabolites into UDP-GlcNAc. When protein synthesis slows, this may spare UDP-GlcNAc and provide a greater opportunity for late branching enzymes (Mgat4, Mga5) to act, thus increasing branching. Indeed, low glucose growth conditions increase surface β1,6GlcNAc-branched *N*-glycans in primary fibroblasts (Cheung et al. 2007).

Low glucose conditions also impair $Glc_3Man_9GlcNAc_2$-pp-dol biosynthesis giving rise to smaller intermediates and incomplete *N*-glycosylation (Konishi and Berk 2003; Shang et al. 2007). The accompanying endoplasmic reticulum (ER) stress can exceed the capacity of calnexin and calreticulin (CNX/CRT), cycling by α-glucosidaseII and ER α-glucosyltransferase, and protein folding. Improperly folded glycoproteins bind ER degradation-enhancing α-mannosidase-like protein (EDEM), and are transferred to the cytoplasm (reviewed in Helenius and Aebi (2004)). ATF6, IRE1, and PERK are ER transmembrane glycoproteins and mediators of the ER stress response (reviewed in Schroder and Kaufman (2005)). ATF6 has three conserved *N*-glycosylation sites and their occupancy inhibits the transit of ATF6 from the ER to Golgi and nucleus, where an ATF6 peptide stimulates stress response genes. Underglycosylation of ATF6 may weaken a lectin–glycoprotein interaction in the ER and mobilize ATF6; thus, N-X-S/T occupancy on ATF6 appears to be a sensor for ER stress (Hong et al. 2004). Activated PERK inhibits general translation by phosphorylating the translation initiation factor eIF2α, and this allows $Glc_3Man_9GlcNAc_2$-pp-dol and *N*-glycosylation levels to recover. Therefore, low glucose ER stress regulates protein flux through the ER maintaining homeostasis in $Glc_3Man_9GlcNAc_2$-pp-dol and protein folding (Shang et al. 2007). Similarly, low glucose also slows glycoprotein flux through the *medial*-Golgi increasing UDP-GlcNAc and *N*-glycan branching, which supports lattice retention of receptors mediating growth–arrest. In cancer cells, rapid growth and poor blood supply may contribute to ER stress while ROS-dependent activation of PI3K promotes survival (Bi et al. 2005).

In nontransformed cells under low glucose conditions, slower translation and trafficking through Golgi to the surface are expected to favor lattice avidity, thus autocrine TGF-β/Smad signaling. In oncogene-transformed cells, increased protein synthesis, metabolism, and overexpression of late branching enzymes promotes growth autonomy (Figure 4). However, it is possible that "nontransformed" dynamics can be restored to ER (by reducing TORC1/SK6), Golgi pathway ultrasensitive (by reducing Mgat5 and increasing Mgat1), and driving hexosamine flux (by GlcNAc supplementation). The objective would be to concomitantly increase extracellular receptors that drive tumor suppressor pathways (TGF-β/Smad, cadherins, integrins). Unlike restoring a single tumor suppressor such as p53 (Xue et al. 2007), the *N*-glycan pathway regulates multiple surface glycoprotein in a coordinated manner. GSK3 is an interesting candidate as a modifier or hexosamine/Golgi/lattice, which is constitutively active and inhibited by RTK/AKT and WNT signaling (Kockeritz et al. 2006). GSK3 inhibits glycogen synthesis as well as ATP citrate lyase, an enzyme upstream of fatty acids, cholesterol and dolichol, and possibly *N*-glycosylation (Figure 2). Inhibitors of GSK3 enhance early processing and β1,6GlcNAc-branched *N*-glycans while slowing growth of HeLa tumor cells (Rodriquez and Dennis, unpublished).

Evolutionary considerations

TORC1/S6K and GSK3 pathways are conserved in all eukaryotes, whereas *N*-glycan remodeling displays marked evolutionary change. Mature *N*-glycans in *Arabidopsis*, *C. elegans*, and *Drosophila* are mannose-terminated structures, and very little branching occurs beyond mono- and a trace of biantennary (Zhu et al. 2004). Curiously, a specific β-*N*-acetylhexosaminadase activity in these animals removes most of the GlcNAc residues added by Mgat1, which blocks further branching (Gutternigg et al. 2007). Considering the relative affinities of Mgat enzymes for UDP-GlcNAc, monoantennary *N*-glycan levels may depend largely on competition between Mgat1 and β-hexosaminidase, while UDP-GlcNAc supply can be limiting for bianntennary (Km ∼1 mM for Mgat2 and <0.1 mM for Mgat1) (Figure 1B). In *C. elegans* and *Drosophila*, *N*-glycan branching may be more dependent on developmental regulation of Golgi enzymes and less on UDP-GlcNAc metabolism (Figure 4). Golgi β-*N*-acetylhexosaminadase activity is low in mammalian cells implying an important divergence toward regulation by metabolite flux coupled with an expansion of the branching pathway (i.e., more Mgat genes). As described above, multistep ultrasensitivity is critical to differential regulation of high- and low-multiplicity glycoproteins by the hexosamine/Golgi/lattice (Lau et al. 2007). Indeed, vertebrates show expansion of gene families encoding lower multiplicity receptor including those for TGF-β/BMP and Ephrins.

Mutations that disrupt planar cell polarity in *Drosophila* lead to epithelial hyperplasia (similar phenomena as EMT), suggesting that tissue organization and adhesion junctions regulate cell division (Leevers and McNeill 2005). Polarity is dependent on interactions between the Golgi protein Four-jointed (Fj) and transmembrane glycoprotein receptors that establish long-range directional signals (Casal et al. 2006). The Golgi is generally polarized for directional exocytosis, and it is possible that Fj has a role in directional trafficking of glycoproteins based on affinities for endogenous lectins, thus concentrating different glycoforms at the opposite side of the cell. Mutation of Mgat1 in *Drosophila* results in fused beta lobes in the brain, and loss of motility in adult flies, possibly reflecting both developmental and metabolic deficiencies (Sarkar et al. 2006). Curiously, a fused lobe (fdl) mutation has also been identified as the β-hexosaminidase that specifically removes GlcNAc added by Mgat1 (Leonard et al. 2006). Perhaps, Mgat1 products regulate a morphogen gradient or polarity receptors that define the boundary between lobes. $Mgat1^{-/-}$ mouse embryos are also growth impaired and the neural tube fails to close (Ioffe and Stanley 1994; Metzler et al. 1994).

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Future directions

In single cell eukaryotes, growth is constitutive with adequate nutrient and space. Intracellular nutrient-sensing pathways mTOR, GSK3, and PKA are largely conserved from yeast to mammals. However, signaling pathways (e.g., TGF-β, Wnt, Notch, Cadherins) have evolved to integrate growth with differentiation, a critical feature of the metazoan body plan. The expansion of genes encoding *N*-glycan branching enzymes in the evolution of larger and longer lived metazoans may serve to integrate metabolism with growth and tissue renewal in adults. Cancer mutations commonly relieve growth restraints, but restoring these suppressor pathways (e.g., TGF-β, cadherins) has great potential for anticancer therapy. Receptor kinases that mediate arrest and differentiation tend to have low multiplicity, and their dependency on *N*-glycan branching for surface residency is overcome by oncogenesis-driven membrane remodeling. We suggest that "nontransformed" dynamics can be restored by jointly manipulating protein synthesis, *N*-glycosylation, and Golgi remodeling to change the surface distribution of glycoproteins involved in growth and arrest.

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Conflict of interest statement

None declared.

Abbreviations

bFGF, fibroblast growth factor; BMP, bone morphogenic protein; EDEM, ER degradation-enhancing α-mannosidase-like protein; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; fdl, fused lobes; LMW-PTP, low-molecular-weight protein tyrosine phosphatase; NOX, NADPH-dependent oxidase; (PtdIns(3,4,5)P_3), phosphatidylinositol-3,4,5-trisphosphate; PTPs, phosphotyrosine phosphatases; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; SA, sialic acid; siRNA, small interfering RNA; TβR, transforming growth factor-β receptors.

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