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Galectins as Adaptors:
Linking Glycosylation and Metabolism with Extracellular Cues

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Abstract

Galectins interact with *N*-acetyllactosamine (LacNAc) epitopes in transmembrane glycoproteins at the cell surface in a multivalent manner forming a "lattice." The term "galectin lattice" was first used to describe the impact of galectin-3 on immune synapse formation, T cell activation and autoimmunity (Demetriou *et al.* (2001) Nature **409**, 733). The galectin lattice displays rapid exchange of binding partners or stochastic-binding, thereby acting as an intermediary between free diffusion of glycoproteins and stable complexes in the membrane. This includes (i) slowing diffusion and loss of receptor and transporters to coated-pit endocytosis and/or caveolin domains, (ii) slowing the integration of transmembrane phosphatases with signaling microdomains and (iii) promoting turnover (*i.e.*, opposing stability) of cell-cell and focal adhesion complexes. The lattice model classifies galectins as adaptors of glycoprotein functions; regulating their localization, trafficking and thereby activity thresholds. The lattice model has been validated in immune regulation, cell adhesion and motility, and glucose homeostasis in mice. Here we review physical attributes of galectins and their *N*-glycan ligands and apply logical inference, coupled with convergence of biochemical, cell biology and genetic evidence that provide a strong Bayesian probability for greater utility of the lattice model.

A. Conserved but Ubiquitous

Galectins (Gal) are a family of proteins characterized by a ~130 amino acid carbohydrate-recognition domain (CRD) that binds *β*-galactosides (1, 2), ligands found widely and at high density in the glycoprotein products of the secretory pathway (3, 4). The galectin gene family expanded early with the radiation of multicellular eukaryotes (5), along with the genes encoding Golgi *N*-acetylglucosaminyltransferases and *β*-galactosyltansferases, which generate LacNAc epitopes. Galectins are classified into the single CRD group (Gal-1, -2, -5, -7, -10, -11, -13), tandem-repeat (Gal-4, -6, -8, -9, and -12). Gal-3 is unique, with a CRD and a ~120 amino acid N-terminal proline- and glycine-rich domain (6) which is intrinsically disordered (ID) and self-associates in a highly dynamic manner (7), with functionality similar to other low-complexity protein domains that mediate multivalent binding, and phase transitions forming liquid droplets in the cytoplasm (8–10). With specificity for the ubiquitous *β*-galactosides and self-association, galectins have the potential to crosslink transmembrane glycoproteins and form dynamic planar "lattices" with liquid droplet properties (Fig. 1) (reviewed in (11)). Gene duplication and evolutionary pressures have often resulted in divergent binding partners and activities within gene families. However, galectin CRD domains all appear to bind *β*-galactosides with varying affinities; representing a highly conserved functionality in metazoans. There was also a 4-5 fold expansion of potential ligands in early metazoan evolution; genes encoding glycoproteins involved in morphogenesis (12). Galectin binding to *N*-glycans does not require contact with the underlying peptide sequence of the glycoprotein, but rather is dependent on the local concentrations of LacNAc ligands, discussed further below (13). This remarkable conservation of binding specificity suggests a functional imperative, but the ubiquitous distribution of the LacNAc sites, begs the question of how galectins contribute to specific cellular functions.

B. Multivalency

CRD domain affinities for the LacNAc disaccharide [Gal β1-3/4GlcNAc] are in the 100 uM range (14) for Gal-1 and Gal-3, but binding avidity is greatly increased when density of LacNAc units are increased by multi-branched *N*-glycans, poly-LacNAc extension, and to large extent, by multiple *N*-glycan sites per glycoprotein, as suggested much earlier (15, 16). Galectin family members differ in affinities for LacNAc with α2-3 or α2-6 sialic acid and fucose substitutions (17) (See review by Iwaki and Hirabayashi in this series). The tissue-specific expression of enzymes catalysing branching and extension may shift galectin binding toward certain subsets of glycoproteins, and favour different
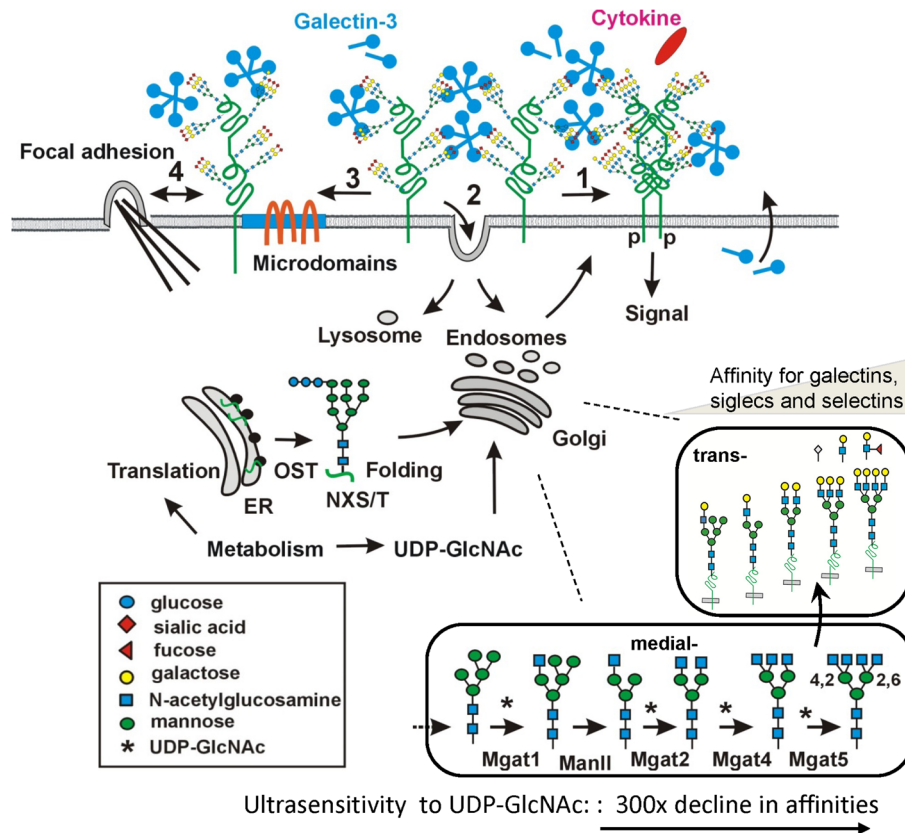
Fig. 1. _N_-glycan branching, galectin-lattice and regulation by metabolism. Galectins bind to _N_-acetyllactosamine (LacNAc) epitopes in Golgi remodeled _N_-glycans Affinities increase with ligand density driven by branching, extension and the number of NXS/T sites in glycoprotein sequences. Galectins gain access to the cell surface by a non-classical secretion mechanism. For simplicity, Gal-3 is shown as an NTD-NTD bound pentamer, which is only one of many possible microstates for self-association in the lattice. NMR and small-angle X-ray scattering analysis of Gal-3 self-association reveals stochastic binding, with many microstates states for complex formation (7). Gal-3 crosslinks transmembrane glycoproteins at the cell surface forming a lattice or liquid-droplet state that serves as an intermediate between stable complexes and free diffusion thereby; (1) inhibits clustering and precocious signaling by receptor tyrosine kinases (2), slowing loss of receptors to endocytosis (3), competing for receptor interaction with caveolae, Cav1 scaffolds and other microdomains, and (4) promotes turnover at cell-substratum and cell–cell adhesions. Galectin ligands are generated by glycosylation in the secretory pathway. Oligosaccharyltransferase (OST) modifies NXS/T(X≠P) sites in the lumen of the rough endoplasmic reticulum (ER). The medial Golgi _N_-glycan branching pathway is multistep ultrasensitivity to increasing UDP-GlcNAc; resulting in a switch-like increase in tri- and tetra-antennary _N_-glycans, thereby affinity for galectin binding.

members of the galectin family. However each glycoprotein is a population of glycoforms, with heterogeneity of glycan structures at each _N_-glycosylation site. The number of _N_-glycosylation sites [NXS/T(X≠P)] is encoded in the sequence, but positions within the protein fold may influence the accessibility to Golgi remodeling enzymes (18, 19). Golgi _N_-acetylglucosaminyltransferases are also sensitive to metabolite supply to the hexosamine biosynthesis pathway (HBP) and flux of UDP-GlcNAc into the Golgi (20, 21).

C. Background to the Lattice Model

The term "galectin lattice" was first used to describe the Golgi _N_-acetylglucosaminyltransferase V (Mgat5) mutation on immune synapse formation, T cell activation and autoimmunity in mice (22). A number of earlier observations lead to this milestone, and the lattice model of galectin function. In the early 1980s, JW Den-

nis and RS Kerbel isolated and characterized lectin-resistant mutants from a metastatic lymphoma cell line, in an effort to assess the function of protein glycosylation in cancer growth and metastasis. Loss-of function mutations were selected in toxic concentrations of plant lectins (_e.g._, wheat germ agglutinin) (23). We found that mutation of the Golgi UDP-galactose transporter reduced solid tumor growth and lung colonization, and interestingly, tumor cell—host cell fusion events suppressed the recessive mutation and restored metastasis (24). Moreover, ectopic restoration of galactose to glycoproteins at the cell surface with UDP-galactose and recombinant β1-4 galatosyltransferase also rescued organ colonization, suggesting that LacNAc plays a role in invasion and growth of blood born tumor cells (25). In these experiments, LacNAc was generated at the cell surface and not available for further modification in the Golgi. At this time, galectins were emerging as likely

candidates for interactions with LacNAc, and particularly Gal-3 which was reported to be over-expressed in cancer (26–28). Oncogenic transformation of cultured fibroblasts increased the activity of tri- and tetra-antennary branching enzymes in the Golgi *N*-glycan remodeling pathway (29–31). It also became clear that branched *N*-glycans were the major binding sites on cultured mammalian cells. The Lec1 (Mgat1) mutation in CHO cells eliminates LacNAc in *N*-glycans, and reduced Gal-1, -3 and -8 binding to the cells by ~90%; a similar reduction was observed with Lec8 mutant cells (UDP-Gal transport) which prevents galactose addition more broadly, on *N*- *O*- linked glycans and glycolipids (32). Mutation or shRNA knockdown of Golgi *N*-acetylglucosaminyltransferase I or V (encoded by Mgat1, Mgat5) inhibited cell migration as well as growth and metastasis in mice (33, 34). With the availability Mgat5-deficient mice and polyomavirus middle T transgene (MMTV-PyMT) cancer model (34), IR Nabi's group led studies revealing phenotypes in focal adhesion dynamics, cell motility and organization of extracellular matrix (35–37). We developed a computational model for regulation of growth factor receptor trafficking and signaling by *N*-glycan remodeling and the galectin lattice (20, 38). In parallel, M. Demetriou's team studied the role of *N*-glycan branching and metabolism in autoimmunity, and in the process validated the galectin lattice with cellular, biochemical and genetic approaches in mice and humans (39).

Mutation of Golgi branching *N*-acetylglucosaminyltransferases reduces LacNAc content in *N*-glycans affecting many glycoproteins at the same time, presumably resulting in complex phenotypes (20, 40, 41). This is more than inference based on phenotypes of the knockout mice. We reported that tumor cells from Mgat5$^{-/-}$ MMTV-PyMT mice (34) were less responsive to multiple growth factors including epidermal growth factor (EGF), fibroblast groth factor (FGF), platelet derived growth factor (PDGF) and transforming growth factor (TGF-β) (38), as well as β1 integrin-dependent signaling (36, 37, 42). We confirmed that *N*-glycans on EGF and TGF-β receptors were ligands for Gal-3, and binding was weaker in Mgat5$^{-/-}$ cells resulting in loss at the receptors from the surface and accumulation in the endosomes. Cytokine signaling could be rescued by (**i**) restoring Mgat5 expression, (**ii**) inhibition of constitutive endocytosis (38), (**iii**) GlcNAc supplementation which increases UDP-GlcNAc and activity of enzymes earlier in the branching pathway (20), (**iv**) adaptive changes in the cells including loss of caveolin-1 expression, which is a negative regulator of EGFR sensitivity (20, 43), and (**v**) altered oxidative phosphorylation allowing constitutive signaling by reactive oxygen species (ROS)-dependent inhibition of phosphatase-and-tensin-homolog (PTEN) and other phosphatases (44).

With these observations, our goal became a systems-level model of cell surface glycoproteins regulated by galectins and *N*-glycans (20). This is contrary to most of the galectin literature to date, where the identification of galectin-specific interactions is the goal. This reductionist approach has diminishing returns in the current research environment where multivalent interactions and phase transition systems are being observed widely (11).

D. Lattice Model

Most glycosylated NXS/T(X≠P) sites are occupied with a biosynthetically-related distribution of *N*-glycan structures. The *N*-glycans can be ordered by LacNAc units and affinities for specific galectins (20), based on available data (14) (See review by Iwaki and Hirabayashi in this series). *N*-glycosylation begins in the ER where the glycan portion of Glc$_3$Man$_9$GlcNAc$_2$-pp-dolichol is co-translationally transferred to Asn at NXS/T(X≠P) sites in the nascent protein. After the removal of glucose and mannose residues, the *N*-glycans are modified in the medial Golgi by *N*-acetylglucosaminyltransferases (encoded by MGAT1, MGAT2, MGAT4a,b,c, MGAT5). The affinity for UDP-GlcNAc declines ~300 fold, from Mgat1 to Mgat5; thus the pathway to tri- and tetra- antennary products is ultrasensitive to UDP-GlcNAc (Fig. 1) (20). The GlcNAc antennae are substrates for β1,4galactosyltransferases, which generates LacNAc antennae and then extended in the trans Golgi to varying degrees as noted above (14). Heterogeneity at each site is due in part to competition between enzymes for acceptor substrates (45), nucleotide-sugar levels (20), as well as cooperative association between enzymes (46), and transit rates of glycoprotein substrates in the Golgi (47). Glycoproteins with *n* posttranslational modification (PTM) sites have an exponential number (n$^{\mathbf{X}}$) of possible isoforms (48), where developmental and environmental conditions determine the cumulative output $\Sigma p_{i…x}$ of the Golgi pathway (49); generating too many glycoforms to evaluate individually (50). However, the glycoform distribution can be estimated based on a knowledge of Golgi pathway activity and affinities of glycans for galectins (14), and used to compute the relative changes in glycoforms, their interaction with galectins.

We constructed an Ordinary Differential Equation (ODE) model with estimates of intracellular enzyme concentrations and kinetic parameters from the literature as outlines (20). We perturbed parameters *in silico* and experimentally, changing UDP-GlcNAc supply, branching enzyme activities, trafficking of substrate glycoproteins (Fig. 2A). The results confirmed that ultrasensitivity (Hill coefficient, Hn) was distributed over multiple reactions; requiring the ~300 fold decline in affinity for UDP-GlcNAc from Mgat1 to Mgat5, and is a robust property of the pathway. Importantly, the model predicts that a gain of Mgat1 activity should reduce the availability of UDP-GlcNAc to Mgat4 and Mgat5,
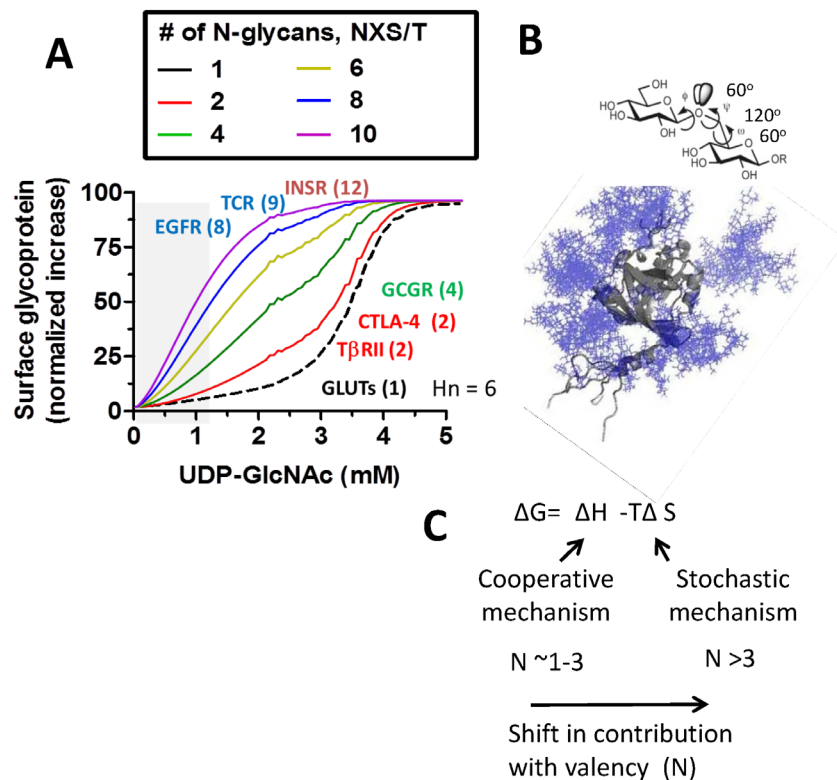
Fig. 2. The functional state of the galectin lattice is a dynamic ensemble. (A) Simulation of HBP-dependent regulation of surface levels of receptors by the galectin lattice in tumor cells using an ordinary differential equation model. Endocytosis was modeled as the major lattice-opposing force. The degree of ultrasensitivity (Hill coefficient) to increasing UDP-GlcNAc/N-glycan branching is inversely proportional to NXS/T N-glycan site multiplicity (modified from Lau. *et al* (20)). Ultrasensitivity of the N-glycan branching pathway to UDP-GlcNAc allows sequential enrichment of receptor in the lattice based on NXS/T multiplicity, thereby an order of opposing signals, for example growth then arrest. (B) N-glycan and glycoprotein microstates: Small-angle X-ray scattering (SAXS) structural data applied to all-atom modeling of five N-glycan chains on α1-acid glycoprotein (183 aa), an overlay of the top five models in the ensemble (modified from Guttman *et al.* (63)). (top) Rotational angles in the three bond of 1–6 linked disaccharide. (C) The flexibility of glycan polymers, multivalency and galectin affinities that are tuned for dynamic "jumping" between sites (*i.e.*, stochastic binding—with microsecond $T_{1/2}$) allows for a large entropic contribution to the lattice structure (modified from (93)).

thus depleting the higher-affinity galectin-3 ligands (tri- and tetra-antennary), while increasing lower-affinity bi-antennary. This was confirmed in non-transformed cells, while increased expression of Mgat4 and/or Mgat5 in cancer cells suppressed the ultrasensitive response to UDP-GlcNAc (51). Endocytosis and Gal-3 binding were assumed to be non-limiting quantities and modeled as linear processes (20). Therefore, the ODE model could be expanded to include variables accounting for expression and activity of specific galectin family members.

E. Bio-Equivalence

A further blow to those seeking specificity is the remarkable amounts of bioequivalence of glycans generated within and between biosynthesis pathways (13, 20, 40, 52). The first demonstration of bioequivalence was the rescue of surface receptors in Mgat5 deficient cells by increasing LacNAc content in N-glycans through exogenous supply of GlcNAc, which is taken up by fluid phase endocytosis and salvaged into HBP to UDP-GlcNAc (20).

Mgat5 has no gene homologues, but compensating amounts of N-acetyllactosamine branches are produced by supplementing Mgat5$^{-/-}$ cells cultures with GlcNAc, which increases UDP-GlcNAc and the activities of Mgat4a,b,c and poly N-acetyllactosamine extension enzyme, and B3GNT-1,-3 family (20, 53). The Golgi branching enzymes function together as an ordered linear pathway, each transferring GlcNAc from UDP-GlcNAc to specific positions on the N-glycans. The degree of ultrasensitivity is inversely proportional to site multiplicity. For example, the glucose transporters GLUT4 has one N-glycan and titration of GlcNAc in cell culture increased cell surface expression with a switch-like response matching the global increase in tetra-antennary N-glycans (*i.e.*, Hn~10) (Fig. 2A) (20). Insulin release by pancreatic β-cells is also dependent on N-glycan branching on GLUT2 transporter and the galectin lattice (54, 55).

Bio-equivalence was also recently demonstrated between hybrid and complex N-glycans based on their LacNAc content (53). Deficiency of medial Golgi enzyme Mgat2 in T cells trig-
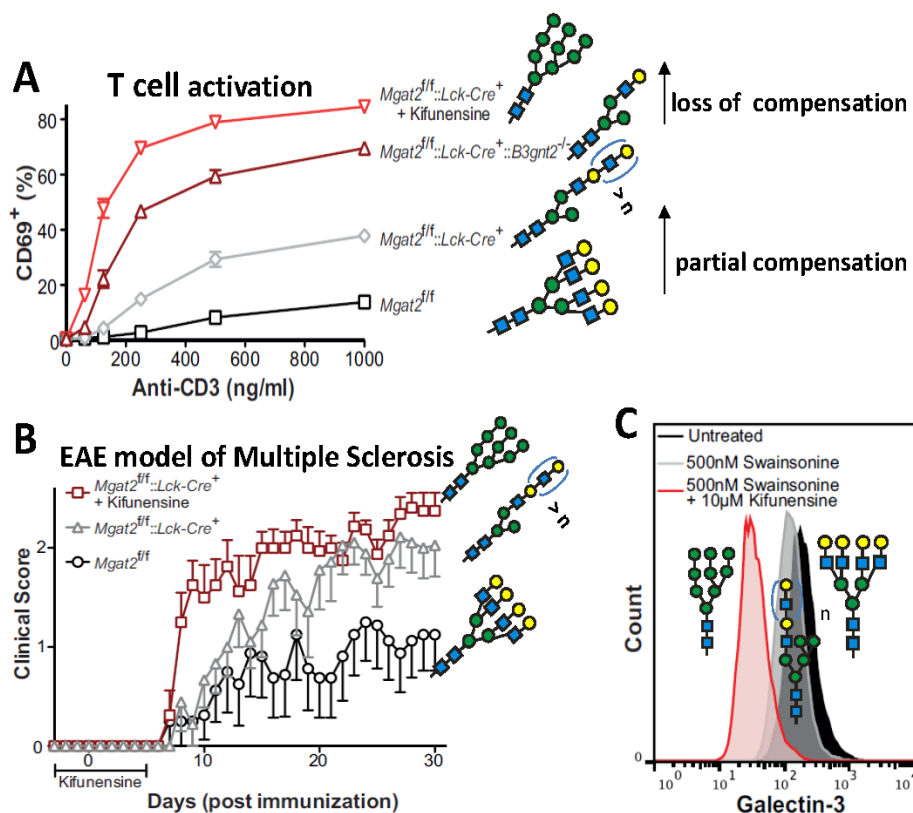
Fig. 3. Bioequivalence of hybrid and complex *N*-glycans in immune cells by rearranging LacNAc units. (A) Mouse T cells of the indicated genotypes and varying placement and number of LacNAc units in *N*-glycans were activated for 24 h with plate bound anti-CD3 and analyzed for CD69 expression by flow cytometry, gating on CD4^{+} cells. Where indicated, mice were pre-treated for 3 days with 0.2 mg/mL kifunensine in the drinking water followed by 10 μM kifunensine during culture, thereby preventing placement of LacNAc in *N*-glycans. (B) Autoimmune encephalomyelitis (EAE) was induced in age-matched female C57BL/6 mice of the indicated genotypes resulting in poly-LacNAc extended hybrid or branched complex *N*-glycans were treated with or without kifunensine in the drinking water at 0.2 mg/mL from day −3 to 5, with day 0 indicating the time of immunization (*n*=9 per group) and assessed daily for clinical signs of EAE. (C) Jurkat T cells were treated as indicated for 72 h in culture and analyzed for galectin-3 binding by flow cytometry (modified from (53)). Mannosidase II/IIx inhibition by swainsonine induces hybrid *N*-glycans with poly-LacNAc extension, which is blocked by mannosidase I inhibition with kifunensine.

gered poly-LacNAc extension of the single GlcNAc branch in hybrid *N*-glycans by the *trans*- Golgi *β*3GNT enzymes, resulting in bio-equivalence to bi- and tri-antennary structures. Remarkably, the data suggested poly-LacNAc extension is triggered by unused UDP-GlcNAc fluxing from the *medial* to *trans* Golgi as a result of hypo-active medial Golgi branching enzymes, thereby enhancing *β*3GNT enzyme activity. In this manner, evolutionary-driven placement of branching enzymes in the medial Golgi *versus* *β*3GNT extension enzymes in *trans*-Golgi provides a fail-safe mechanism to maintain LacNAc content within *N*-glycans. Bio-equivalence plays a critical role in regulating the sensitivity of T cells to autoantigens and prevention of autoimmunity in mice (Fig. 3A–C).

Recently, signal peptide peptidase-like 3 (SPPL3), an intramembrane-cleaving aspartyl protease of the GxGD type, was shown to release the ectodomains of type II membrane proteins, notably Golgi enzymes including *N*-acetylglucosaminyltransfer-ases (MGAT5, MGAT2, B3GNT-1,-3) *β*-1,4 galactosyltransferase 1 (B4GALT1) and *α*-mannosidase 2A1 (MAN2A1) (56, 57). Reduced expression of SPPL3 results in a hyper-glycosylation phenotype, whereas elevated SPPL3 expression causes hypoglycosylation, and may be associated with conditions of metabolic stress. Interestingly, SPPL3 targets multiple Golgi enzymes, consistent with a requirement to regulate *N*-glycan redundancies and bioequivalence at a cellular level.

F.   Lattice Dynamics

The first demonstration of galectin lattice dynamics was the use of exogenous soluble lactosamine to disrupt the interaction of endogenous Gal-3 with the T cell-receptor (TCR) (22). Soluble lactosamine phenocopied the Mgat5 deficiency, enhances both TCR clustering and signaling at the immune synapse (58). This demonstrated for the first time that *N*-glycan branching regulated the adaptor function of galectins, rather than the prevailing dogma

that *N*-glycans alter function *via* changes in protein structure or ligand binding affinity. Galectins were subsequently shown to preferentially bind glycoproteins on surfaces rather than in solution, the former driven by a threshold concentration of multivalent ligands (59). Tandem-repeat galectins readily cross-link glycoproteins, single CRD galectins can dimerize as exemplified by Gal-1 (59). For Gal-3, CRD binding to multivalent glycoproteins promotes the intrinsically-disordered N-terminal domain (NTD) to self-associate *via* amino acid residues ~20–100, accompanied by NTD binding to a surface on the CRD which is not involved in carbohydrate recognition (7, 60, 61). Gal-3 mixed with a bivalent *N*-glycan ligand *in vitro* at optimal concentrations, undergoes phase transition to a liquid-droplet state, which is NTD dependent and can be inhibited by monovalent β-galactosides (62). Factors that contribute to liquid-droplet state of the lattice include multivalency, irregular geometry and entropic contribution to binding (Fig. 2C). In live cells, the lattice is planar liquid-droplets, held in proximity to the membrane by transmembrane glycoproteins. The diversity of glycoproteins contribute to a disordered geometry and heterogeneity of the galectin lattice (11). Furthermore, *N*-glycans generally extend away from the protein surface and display the entropy of a flexible polymer (63) (Fig. 2B, C). The Gal-3 CRD also displays conformational entropy which contributes favorably to the multivalent stochastic binding of ligand (64, 65). Gal-3 and Gal-9 cross-linking of transmembrane glycoproteins can slow lateral diffusion of freely diffusing glycoproteins as measured by fluorescence recovery after photobleaching (FRAP) (43). Manipulating galectin levels, *N*-glycan branching activities or endocytosis alters
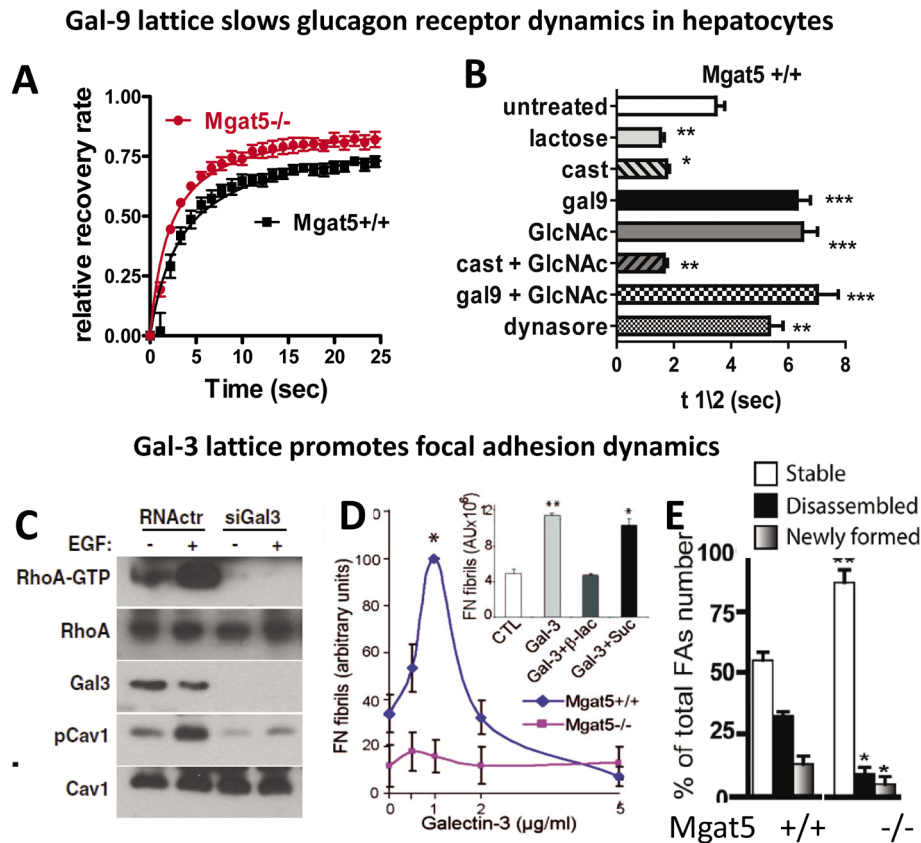
Fig. 4. **Galectin lattice regulates the dynamics of hepatic glucagon receptor and focal adhesions.** (A) Time course of fluorescence recovery after photobleaching (FRAP) of GFP-tagged glucagon receptor in primary mouse hepatocytes ($n=5$). (B) Half-life ($T_{1/2}$) for recovery of GFP-tagged glucagon receptor in primary hepatocytes pre-treated as indicated with either 30 mM lactose, 100 μM castanospermine, and 20 mM GlcNAc; ($n=5$ independent experiments, 6–10 cell bleached/experiment) (modified from (40)). Lack of interaction with the galectin lattice in Mgat5$^{-/-}$ hepatocytes increases receptor mobility. (C) MDA-MB-231 cells were transfected with control (ctr) or Gal3-targeted (Gal3) siRNA for 48 h followed by treatment with EGF for 5 min; lysates were subjected to Rhotekin-RBD pull down for activated RhoA (RhoA-GTP) relative to the total amount of RhoA, and western blotted for Gal-3, Cav1 and Y14-phosphorylated pCav1 levels (modified from (66)). (D) Fibronectin polymerization by Mgat5$^{+/+}$ and Mgat5$^{-/-}$ mammary tumor cells plated in serum-free medium supplemented with 0, 0.5, 1, 2, and 5 μg/mL recombinant galectin-3 (modified from (36)). (E) Mgat5$^{+/+}$ and Mgat5$^{-/-}$ mammary tumor cells were transfected with FAK-GFP and imaged every 30 s for 30 min. Overlay of FAK-GFP images at time 0 (red) and 30 min (green) allowed quantification of disassembled (red), stable (yellow), and newly formed (green) FAs (modified from (37)). The galectin lattice therefore promotes focal adhesion signalling, turnover and matrix re-modeling.

FRAP half-life of glucagon receptor at the cell surface in a manner consistent with the lattice model (40) (Fig. 4A, B). Exogenous galectin promotes the turnover of β1-integrin-dependent focal adhesion signaling motility as well as fibronectin polymerization (Fig. 4C–E). These responses to increasing galectin concentrations were non-monotonic. The titration of recombinant Gal-3 stimulates cell migration and fibronectin remodeling (36) at an optimal concentration, above which Gal-3 inhibits (36, 66, 67). The biphasic response to Gal-3 and Gal-8 (68, 69) is analogous to precipitation responses to divalent antibody, and highlighting the importance of galectins as multivalent adaptors. Lattice dynamics have also been measured by fluorescence resonance energy transfer (FRET) (70), and single-particle tracking (71).

G.   Emergent Properties of the Galectin Lattice

How does the galectin lattice provide a selective advantage to organisms? In proliferating and motile cells there is often high rates of membrane turnover, and the galectin lattice opposes receptor internalization (38), but does so unequally in proportion to NXS/T(X≠P), an evolved feature of each receptor (20). For example, epidermal growth factor receptor (EGFR) with eight occupied sites, stimulates metabolism including UDP-GlcNAc biosynthesis, and this ultimately recruits TGF-β receptors with two N-glycans into the lattice allowing increased autocrine TGF-β/Smad2/3 signaling and suppression of cell proliferation (20). The ODE model and experimental validation agreed well in this and other examples where differences in NXS/T(X≠P) number between receptors that signal in opposite directions creates a UDP-GlcNAc-dependent delay in their recruitment to the lattice. Receptors with low site number are recruited at a later stage in the titration of UDP-GlcNAc and branching. Examples include T cell activation followed by arrest (39) and hepatic glycolysis balanced against gluconeogenesis (40, 72). Thus, N-glycan biosynthesis and biophysical properties of the galectin lattice provide a selective advantage to animals in the form of tunable and ultrasensitive stimulus-response relationships.

The galectin-lattice model has been validated in several biological settings. In T cells, the lattice suppresses T cell receptor signalling to control antigen-induced activation and positive selection in the thymus to promote self-tolerance and prevent autoimmunity (22, 73, 74). In humans, single nucleotide polymorphisms (SNPs) in MGAT1, MGAT5 and CTLA4 genes are associated with Multiple Sclerosis (39, 75). Gain-of-activity SNPs in the coding sequence of Mgat1 increase expression and when coupled with ~300 fold higher affinity of Mgat1 for UDP-GlcNAc relative to Mgat5; this reduces UDP-GlcNAc availability to Mgat5, which suppresses LacNAc content and galectin-dependent regulation of T cells. The Mgat1 SNPs interact with a SNP in CTLA-4 that reduces N-glycan

site occupancy, for a combined increase in Multiple Sclerosis risk. Together, they decrease lattice recruitment of CTLA-4 to the cell surface, thereby delaying its action as a negative regulator of T cell proliferation (20). CTLA-4 levels are rescued by GlcNAc supplementation in tissue culture, and *in vivo* oral GlcNAc also suppresses experimental autoimmune disease in mice and rescues signaling consistent with the model (76, 77). Oral GlcNAc in MS patients is currently in clinical trials led by Michael Demetriou. These observations and a genome-wide evolutionary analysis of NXS/T(X≠P) sites is consistent with selective pressures on subsets of glycoproteins in opposing directions (*i.e.*, high or low site number) (78, 79). This is an emergent property associated with the evolution of Golgi N-glycan processing and galectins as adaptors of receptors regulating opposing pathways.
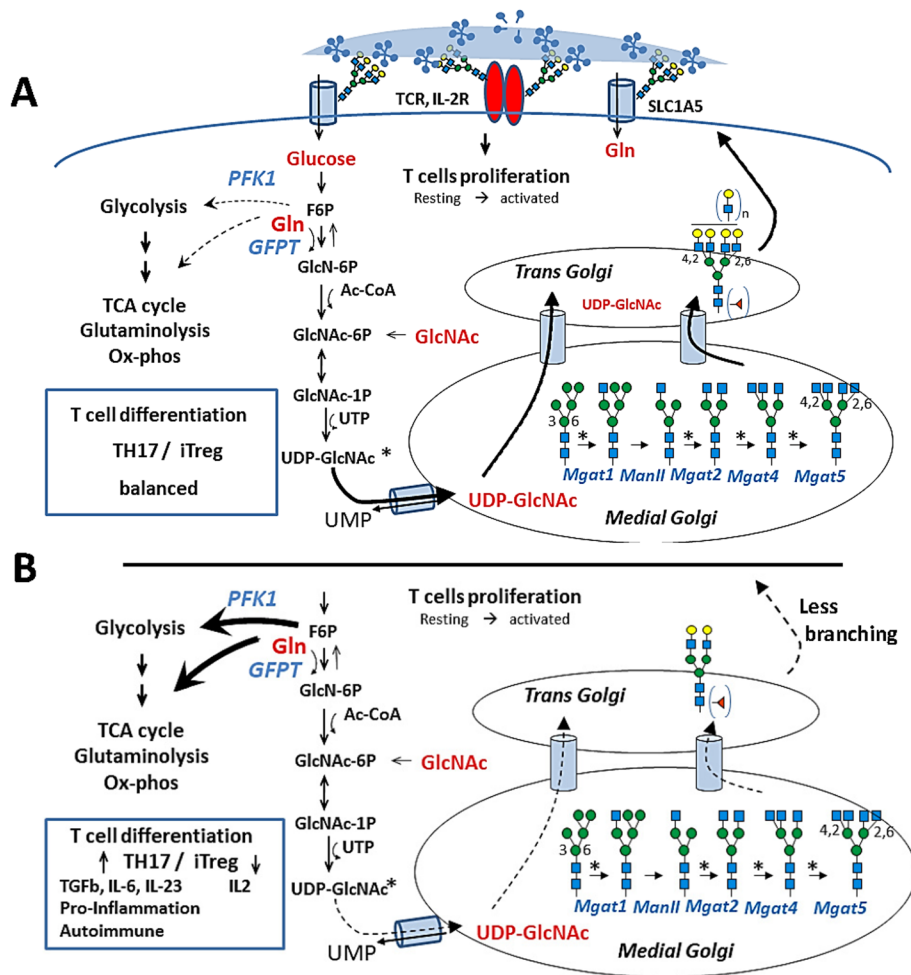
H.   Metabolic Regulation of the Lattice

Ultrasensitivity of the branching pathway and LacNAc content within N-glycans depends on the production of UDP-GlcNAc, which can be taken advantage of therapeutically through GlcNAc supplementation and salvage into the HBP. However, *de novo* synthesis of UDP-GlcNAc from glucose provides another critical mechanism regulating lattice strength. The HBP directly competes with glycolysis for available glucose (Fig. 5). The most significant metabolic change in cells is a switch from complete oxidation of glucose that produces 36–38 ATP (oxidative phosphorylation) to fermentation of glucose to lactate and generation of only 2 ATP. This switch is induced when oxygen is limiting (anaerobic glycolysis) but is paradoxically also induced in rapidly dividing cells despite sufficient oxygen (aerobic glycolysis), a phenomenon first reported in cancer cells by Otto Warburg over 90 years ago. Aerobic glycolysis is often accompanied by glutaminolysis, where glutamine is converted to α-ketoglutarate for flux into the TCA cycle. Glutamine is required for *de novo* synthesis of UDP-GlcNAc by the HBP, acting as an amine donor in the rate limiting conversion of fructose-6-phosphate to glucosamine-6-phosphate. In blasting T cells, aerobic glycolysis and glutaminolysis cooperatively reduce *de novo* UDP-GlcNAc production by limiting flux of glucose and glutamine into the HPB. This lowers N-glycan branching and lattice strength to drive T cell growth and pro-inflammatory T_H17 over anti-inflammatory T regulatory (iTreg) differentiation by lowering IL-2Rα (CD25) surface levels (52, 77) (Fig. 5). In this manner, aerobic glycolysis and glutaminolysis serve as critical negative regulators of lattice strength, receptor content and cell fate.

I.   Galectins as Adaptors of Glycan Mimics in the Cytoplasm

The primordial function of galectins may have been the ca-

Fig. 5. Metabolic regulation of HBP and *N*-glycan branching. (A) Glucose is metabolized into fructose 6-phosphate, which supplies both glycolysis and the HBP. Oxidative phosphorylation has modest needs for glucose, allowing the HBP to effectively compete with glycolysis for glucose flux to fructose-6-phosphate and thereby drive UDP-GlcNAc biosynthesis, *N*-glycan branching and balanced T cell responses. (B) Rapidly proliferating T cells switch from oxidative phosphorylation to aerobic glycolysis and glutaminolysis. This reduces flux of fructose-6-phosphate and glutamine into the HBP, thereby lowering UDP-GlcNAc biosynthesis and *N*-glycan branching to drive pro-inflammatory T cell responses. Thus, a primary function of aerobic glycolysis and glutaminolysis is to co-operatively limit metabolite supply to *N*-glycan biosynthesis, an activity with widespread implications for autoimmunity and cancer (52). Note Gal-3 as shown is only one of many possible microstates for self-association in the lattice.

pacity to cross-link β-galactoside-containing glycoconjugates. With the subsequent emergence of the galectin gene family in animals, β-galactoside binding activity has been conserved, and unencumbered by covalent linkage with other activities. Galectin CRDs have *not* been found in larger multi-domain proteins, as is common for other PTM-binding folds such as SH2 and PTB phosphotyrosine-binding domains (80). These observations are consistent with the adaptor function regulated in large part by β-galactoside ligand multiplicity and the modest affinities of galectin CRD domains which allow stochastic cross-linking.

However, some CRDs have evolved additional affinities for specific peptide sequences in cytosolic proteins with important adaptor functions. Cytosolic Gal-3 is reported to bind β-catenin, which could be inhibited by lactose (81, 82). Similarly, Gal-3 and

Gal-1 association with pre-mRNA splicing and nuclear ribonucleoprotein complexes are inhibited by lactose (82–84). This suggests that peptide binding occurs at the β-galactoside site in the CRD. The intracellular pathogen *Salmonella Typhimurium* cause vesicle damage, which disrupts lysosomal vesicles and exposes *N*-glycans to the cytosol which act as an "eat-me" signals that recruit galectins (85). While the N-terminal CRD of galectin-8 binds LacNAc, the C-terminal CRD also binds a nine amino acid sequence in NDP52, which in turn, recruits NAP1, TANK, ubiquitin receptors leading to autophagy and suppression of *Salmonella* (86, 87). Lysosomal membrane proteins (LAMP) are major carriers of *N*-glycan chains with poly *N*-acetyllactosamine chains (88, 89). The C-terminal CRD of Gal-8 has evolved to recognize both the peptide and at lower affinity, β-galactosides. The bacteria-associated "eat-

me" signals can be mediated by either Gal-8 or K48- K63-linked ubiquitin chains on the *Salmonella*; either suffices to recruit TBK1, which is essential for cell-autonomous immunity against infections.

J. The Bigger Picture

It is instructive to consider similarities with other multivalent PTM systems (see review (11)). As described above for the galectin lattice (20), the order of substrate activation by titration of PTM enzyme activity and site multiplicity has been described for protein phosphorylation by Cdk1 in the G1/S transition (90) and polyubiquitination by APC\C during exit from mitosis (91–93). The PTM modifiable sites often occur frequently in protein sequences, and sites function together acting as epitopes for binding partners (94, 95). Clustering of modifiable sites in the sequences of authentic protein substrates permits rapid and efficient processive modification. Specificity emerges in large part from the cooperative effects of PTM site clustering and chain formation, while decoy-substrates promote inhibitor ultrasensitivity, thereby tuning the stimulus-response relationships (90, 96). PTM dependent interactions with adaptor proteins are dynamic "jumping" between sites (*i.e.*, stochastic binding—microsecond $T_{1/2}$) (16, 97), with cross-linking that can form lattice or liquid-droplet membrane-less organelles (8–10). Multivalent systems can buffer mutational loss or gain of PTM sites, thereby facilitating evolution of site positions (78, 79, 98). As such, the advantages are based on bioequivalence, multivalent interactions, evolvability of sites and tunable sensitivity of stimulation-response relationships by metabolism.

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