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REVIEW ARTICLE

Assembly, organization and regulation of cell-surface receptors by lectin–glycan complexes

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Galectins are a family of β-galactoside-binding lectins carrying at least one consensus sequence in the carbohydrate-recognition domain. Properties of glycosylated ligands, such as *N*- and *O*-glycan branching, LacNAc (*N*-acetyl-lactosamine) content and the balance of α2,3- and α2,6-linked sialic acid dramatically influence galectin binding to a preferential set of counter-receptors. The presentation of specific glycans in galectin-binding partners is also critical, as proper orientation and clustering of oligosaccharide ligands on multiple carbohydrate side chains increase the binding avidity of galectins for particular glycosylated receptors. When galectins are released from the cells, they typically concentrate on the cell surface and the local matrix, raising their local concentration. Thus galectins can form their own multimers in the extracellular milieu, which in turn cross-link glycoconjugates on the cell surface generating galectin–glycan complexes that modulate intracellular signalling pathways, thus regulating cellular processes such as apoptosis, proliferation, migration and angiogenesis. Subtle changes in receptor expression, rates of protein synthesis, activities of Golgi enzymes, metabolite concentrations supporting glycan biosynthesis, density of glycans, strength of protein–protein interactions at the plasma membrane and stoichiometry may modify galectin–glycan complexes. Although galectins are key contributors to the formation of these extended glycan complexes leading to promotion of receptor segregation/clustering, and inhibition of receptor internalization by surface retention, when these complexes are disrupted, some galectins, particularly galectin-3 and -4, showed the ability to drive clathrin-independent mechanisms of endocytosis. In the present review, we summarize the data available on the assembly, hierarchical organization and regulation of conspicuous galectin–glycan complexes, and their implications in health and disease.

Key words: CD45, galectins, glycan receptors, *N*- and *O*-glycosylation, surface retention, VEGFR2.

INTRODUCTION

Galectins (Gals) are an evolutionarily conserved family of proteins first identified as galactoside-binding lectins and formally defined on the basis of a shared sequence [1]. The consensus sequence corresponds to the CRD (carbohydrate-recognition domain) comprising ~130 amino acids, which interact with certain glycan structures on cell-surface glycoconjugates. In fact, Gals might be considered a reading code for repetition of *N*-acetyl-lactosamine (Galβ1,4GlcNAc; LacNAc) units present in glycosylated ligands. In mammals, 15 Gals have been characterized and numbered according to the chronology of their discovery (Gal-1 to Gal-15) [2]. Gals known so far have one or two CRDs within a single polypeptide chain. The mono-CRD Gals (Gal-1, Gal-2, Gal-5, Gal-7, Gal-10, Gal-11, Gal-13, Gal-14 and Gal-15) can be biologically active as monomers or as homodimers. Bi-CRD Gals (Gal-4, Gal-6, Gal-8, Gal-9 and Gal-12) are active as monomers, but can also form oligomers. Gal-3 is the only representative member of the chimera Gal-type subfamily, containing a long N-terminal collagen-like domain (with proline- and glycine-rich motifs) and a C-terminal domain with a single CRD [3]. Orientation, rotational flexibility, and spacing of the CRDs in Gals also contribute to recognition of specific glycosylated ligands spatially distant from one another

Abbreviations: ADAM15, a disintegrin and metalloprotease 15; BCR, B-cell receptor; Cav1, caveolin-1; C2GnT, core 2 β1,6-N-acetylglucosaminyltransferase; CLIC, clathrin-independent carrier; CNS, central nervous system; CRD, carbohydrate-recognition domain; CTLA-4, cytotoxic T-lymphocyte antigen-4; DC, dendritic cell; EC, endothelial cell; ECM, extracellular matrix; EEA1, early endosomal antigen 1; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial–mesenchymal transition; ERK1/2, extracellular-signal-regulated kinase 1/2; F-actin, filamentous actin; FA, focal adhesion; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FN, fibronectin; Gal, galectin; GEM, ganglioside GM1-enriched microdomain; Glut-2, glucose transporter 2; GSL, glycosphingolipid; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; IFN-γ, interferon γ; IGF, insulin-like growth factor; iGnT, β1,3-*N*-acetylglucosaminyltransferase; IGnT, I β1,6-*N*-acetylglucosaminyltransferase; IL, interleukin; LacNAc, *N*-acetyl-lactosamine; LAT, linker of activated T-cells; Lck, leucocyte-specific protein tyrosine kinase; L-PHA, leucoagglutinin from *Phaseolus vulgaris* ; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mgat, mannosyl-glycoprotein *N*-acetylglucosaminyltransferase; N-cadherin, neural cadherin; Nck, non-catalytic region of tyrosine kinase; PDGF, platelet-derived growth factor; PDI, protein disulfide-isomerase; PNA, peanut agglutinin; PyMT, polyoma middle T; SLP76, Src homology 2 domain-containing leucocyte protein of 76 kDa; SNA, *Sambucus nigra* agglutinin; ST3Gal, α2,3-sialyltransferase; ST6Gal1, α2,6-sialyltransferase 1; Syk, spleen tyrosine kinase; TCR, T-cell receptor; TGF, transforming growth factor; TβR, TGF-β receptor; Treg, regulatory T-cell; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; WASp, Wiskott–Aldrich syndrome protein; ZAP-70, ζ-chain-associated protein kinase of 70 kDa.

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(*cis*) or even on different cell types (*trans*) [4]. In contrast with the rigid homodimer structure of native Gal-1 [5], for example, the two Gal-9 CRDs are on either end of a flexible peptide linker, therefore the tandem repeat Gal-9 CRD is predicted to have greater rotational flexibility than the Gal-1 CRDs in the homodimer; thus Gal-9 may be able to cross-link glycoproteins that are more sparsely distributed or spatially distant on the cell surface, compared with Gal-1 [4]. In addition, bi-CRD Gals including Gal-8 [6] and Gal-9 [7] can multimerize via interactions of the N-terminal CRDs so that higher-order multimers could bind to cell surfaces.

Gals are secreted from cells through a still unresolved mechanism, which does not involve the classical ER (endoplasmic reticulum)/Golgi-dependent secretory pathway [8]. The ECM (extracellular matrix) provides a rich source of saccharide ligands on glycoproteins such as laminin, FN (fibronectin) and vitronectin, which may contribute to maintaining Gal carbohydrate-binding activity. Moreover, ECM glycoproteins can concentrate Gals secreted by surrounding cells [9]. A secreted Gal remains associated to the cell surface through binding to β-galactoside-containing oligosaccharides, typically in complex *N*-glycans and core 2 *O*-glycans on cell-surface glycoproteins, but also in glycolipids as documented for Gal-1, Gal-2, Gal-3, Gal-4, Gal-8 and Gal-9, which have been shown either to interact with glycolipids or to be localized in lipid rafts [10–19]. Monomeric Gals can oligomerize to form homodimers such as Gal-1 [20–22] or pentamers such as Gal-3, i.e. secreted Gal-3 forms multimers via interactions of the N-terminal domain, promoting multivalent binding of the C-terminal CRD to its receptors [23–25]. Gal multimers thereby cross-link glycoconjugates from the cell surfaces producing Gal–glycan complexes, also termed Gal–glycan lattices, which can modulate intracellular signalling pathways and regulate cellular processes such as apoptosis, proliferation, differentiation and migration [26–28]. Brewer's group has demonstrated that bovine Gal-1 can form ordered homotypic complexes composed of identical glycoproteins out of a mixture of glycoproteins in solution [29]. The formation of homotypic complexes is thermodynamically driven, suggesting that these supramolecular structures also occur on the cell surface: in solution, the ability of Gal-1 cross-linking to form homotypic or heterotypic complexes of soluble glycoproteins relies on the number of oligosaccharide ligands per glycoprotein that could bind Gal-1, a phenomenon termed glycoprotein valency [29,30]. Gal–glycan complexes selectively regulate the dynamics of glycosylated binding partners, both proteins and lipids, limiting receptor internalization and maintaining downstream signalling sensitivity. Subtle changes in receptor expression, different rates of protein synthesis, selective activities of Golgi enzymes, concentrations of metabolites that support glycan biosynthesis, density of receptors and protein–protein interactions, altogether dictate the stoichiometry of Gal–glycan cross-linking [16,31,32]. In the present review, we summarize biochemical, biophysical and functional aspects of Gal–glycan complexes, their signalling capacity and the physiological programmes regulated by these signalling clusters.

N- and *O*-glycans as Gal-binding partners

Gals can bind to *N*-glycans, *O*-glycans and glycolipids, usually with affinities proportional to LacNAc and poly-LacNAc content [13]. Thus higher poly-LacNAc extensions and no or low capping by α2,6-sialylation favour Gal binding [33,34]. However, different members of the Gal family may display considerable differences in glycan-binding preferences, a biochemical property that may affect their signalling capacity and function [28,30]. With regard to *N*-glycans, most of the Asn-Xaa-Ser/Thr (N-X-S/T) motifs exposed to the lumen of the endoplasmic reticulum are co-translationally *N*-glycosylated and the medial Golgi-branching Mgat1, Mgat2, Mgat4a/b and Mgat5 (*N*-acetylglucosaminyltransferases I, II, IVa/b and V, respectively) catalyse the glycan branching, which is sensitive to metabolic flux through the hexosamine pathway and by genetic determinants of Golgi activity [32,35,36]. In fact, mannose residues from high-mannose glycans are removed in the *cis*-Golgi, and Mgats initiate the branches in a sequential and ordered manner by adding GlcNAc residues [37] (Figure 1). Mannosyl-α1,6-glycoprotein β1,6-*N*-acetylglucosaminyltransferase (Mgat5 or GlcNAc-TV) catalyses the addition of β1,6-GlcNAc to *N*-glycan intermediates on newly synthesized glycoproteins that transit the *medial*-Golgi [38]. These *N*-glycan intermediates are later elongated in the *trans*-Golgi to produce tri- (2,2,6) and tetra- (2,4,2,6) antennary *N*-glycans, which are extended with LacNAc and poly-LacNAc [39] (Figure 1). Indeed, branched *N*-glycan intermediates are lineally elongated with poly-LacNAc by β1,4-galactosyltransferases and β1,3-N-acetylglucosaminyltransferases (iGnTs), and capped with sialic acid and fucose [32,40,41]. Importantly, Dennis et al. [42] showed that an Mgat5-deficient metastatic tumour cell line was less tumorigenic and metastatic. Later, the authors demonstrated that ablating the *Mgat5* gene in transgenic mice that overexpress the PyMT (polyoma middle T) oncogene markedly reduced mammary tumour development and lung metastasis [43]. Likewise, oncogenic transformation was demonstrated to enhance gene expression of Mgat4, Mgat5 and iGnTs, which reduces the requirement for hexosamine supplementation by supporting higher levels of tri- and tetra-antennary *N*-glycan biosynthesis in tumour cells [40,44,45].

Regarding *O*-glycans, core-type protein glycosylation is initiated in the secretory pathway by the covalent addition of one GalNAc to serine or threonine, then other glycosyltransferases sequentially elaborate the repertoire of *O*-glycan structures to include different core subtypes [46]. Importantly, the core 2 β1,6-*N*-acetylglucosaminyltransferases (C2GnTs) generate core 2 *O*-glycans, widely expressed in mammalian cells. The C2GnTs act subsequent to the core 1 β1,3-galactosyltransferase, which adds a galactose in a β1,3-linkage to the GalNAc-Ser/Thr generating the initial core 1 *O*-glycan disaccharide structure. C2GnTs (C2GnT1, C2GnT2, and C2GnT3) then add GlcNAc in a β1,6-linkage to the GalNAc to initiate what is known as the core 2 *O*-glycan branch [47–49] (Figure 1). Another pathway involves the core 3 β1,3-N-acetylglucosaminyltransferase (C3GnT), which adds a GlcNAc to the unmodified GalNAc to generate a core 3 *O*-glycan; in this case, C2GnT2 can add a GlcNAc in β1,6-linkage to the GalNAc of the core 3 *O*-glycan disaccharide to initiate the formation of a core 4 *O*-glycan. Furthermore, I-branched poly-LacNAc (a unique carbohydrate composed of LacNAc branches attached to linear poly-LacNAc chains) are also common and formed by I β1,6-N-acetylglucosaminyltransferase (IGnT) [50,51] and C2GnT2 [52] (Figure 1).

Functions of Gal–glycan complexes at the cell surface of leucocytes

Gals are highly expressed in leucocytes, mostly in activated T-cells, Tregs (regulatory T-cells), macrophages and DCs (dendritic cells), and regulate key immunological processes such as cytokine production, differentiation, apoptosis and tolerance [53]. For example, in the periphery, Gal-1 promotes selective apoptosis of Th1 and Th17 cells [34], induces IL (interleukin)-10 secretion [54–56], inhibits T-cell trafficking [57] and decreases

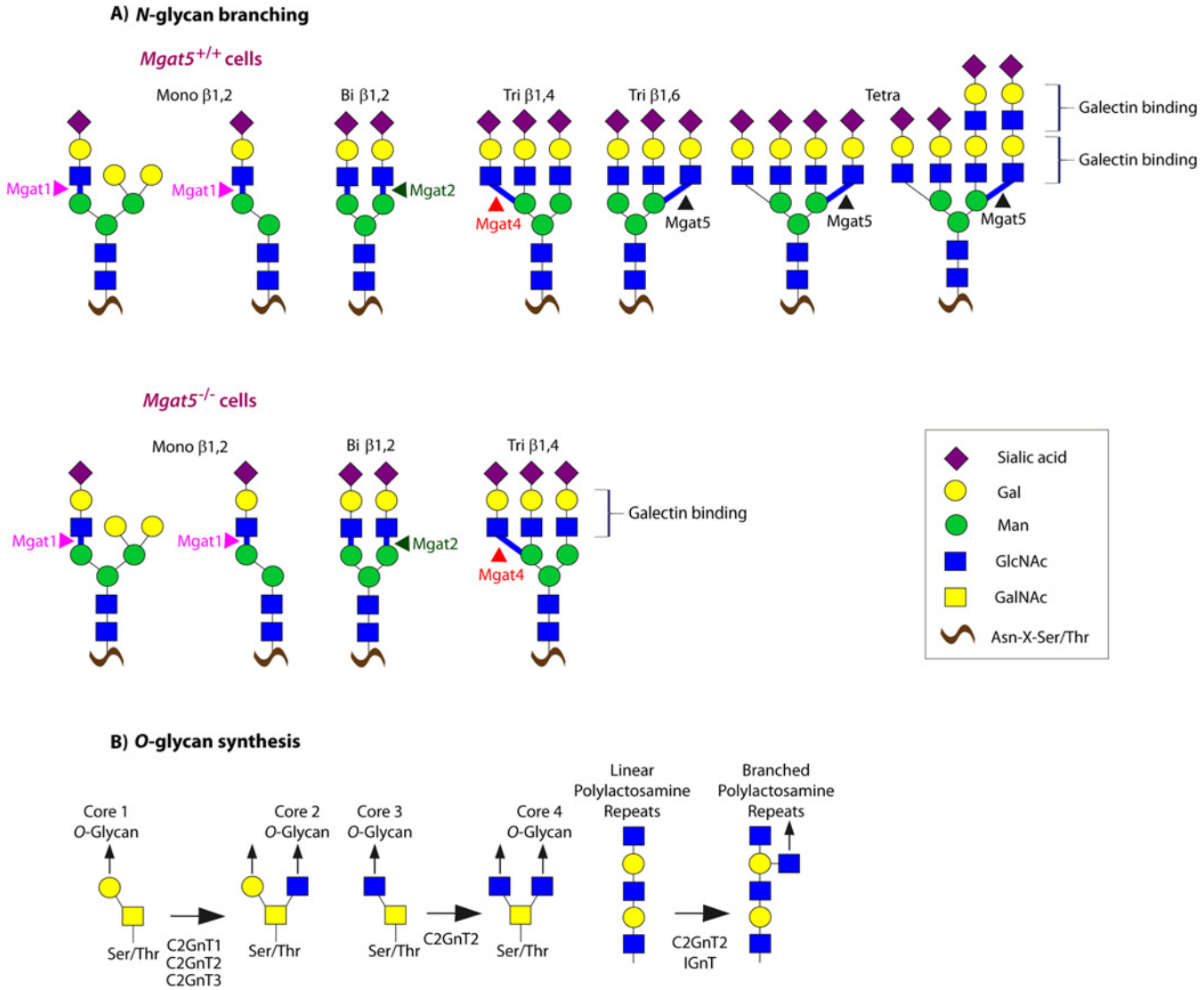

Figure 1 ***N*-glycan branching and *O*-glycan biosynthesis in mammalian cells**

(**A**) The branched *N*-glycans attached at Asn-Xaa-Ser/Thr sites are shown: *Mgat5*$^{-/-}$ cells cannot generate the β1,6GlcNAc branch (black arrowheads) and so they cannot synthesize the most complex types of branched *N*-glycans unlike *Mgat5*$^{+/+}$ wild-type cells. Branching in each antenna has been coloured blue and corresponds to the β1,2GlcNAc, β1,4GlcNAc or β1,6GlcNAc linkages to the mannose core. Mono-, bi-, tri- and tetra-antennary glycans are referred to by the number of branches in an *N*-glycan. Mgat4 catalyses the addition of the β1,4GlcNAc branch to the trimannosyl core, and is represented by red arrowheads. Mgat1 and Mgat2 enzymatic activities are represented by pink and green arrowheads, respectively. Gal binding to LacNAc (Galβ1,4GlcNAc) units is shown. (**B**) *O*-glycan biosynthesis is represented, showing the key activity of C2GnTs: bi-antennary core 2 *O*-glycans are generated when any of the three C2GnTs acts on the core 1 *O*-glycan disaccharide. C2GnT2 can generate core 4 *O*-glycans from core 3 *O*-glycans by adding a GlcNAc to the initiating GalNAc. C2GnT2, in addition to IGnT, also has the ability to generate branched polylactosamine repeats from linear polylactosamine repeats. Distal I-branching (branched repeats of LacNAc) is shown as the GlcNAc transferred to the predistal galactose, the preferential I-branching activity of C2GnT2. IGnT has mainly central I-branching activity, which adds GlcNAc on internal galactoses; the vertical black arrows indicate the potential for further elongation of each branch.

antigen-presenting capacity and nitric oxide production by macrophages [58,59]. Gal-1 also promotes the differentiation of IL-27-producing tolerogenic DCs [60] and favours the expansion of inducible Tregs [61].

Gal-1 interacts with both *N*- and *O*-glycans on various cell-surface glycoproteins as well as in GSLs (glycosphingolipids) including gangliosides [62–68]. Within the immune system, this lectin binds to a wide array of T-cell-surface glycoproteins and glycolipids which serve as counterligands including CD45, CD43, GM1, CD2, CD3, CD4, CD7 [69] and β-integrins [70]. Several T-cell-surface glycoproteins can regulate susceptibility of thymocytes and T-cells to Gal-1-induced death, including CD7 [71], CD43 [72] and CD45 [65,73], which is a major receptor for Gal-1 on T-cells [74]. Strikingly, Gal-1 binds to the glycoprotein receptor CD43, a heavily charged transmembrane sialomucin which bears core 1 *O*-glycans, showing that low-affinity/high-avidity binding to a highly abundant but less preferred glycan ligand Galβ1,3GalNAc is sufficient to induce T-cell death [72].

CD45, a highly glycosylated protein with tyrosine phosphatase activity present in all nucleated haemopoietic cells, is present on different isoforms, which carry *N*- and *O*-glycosylation sites that are regulated during T-cell development, activation and differentiation [74]. In fact, the extracellular domain of

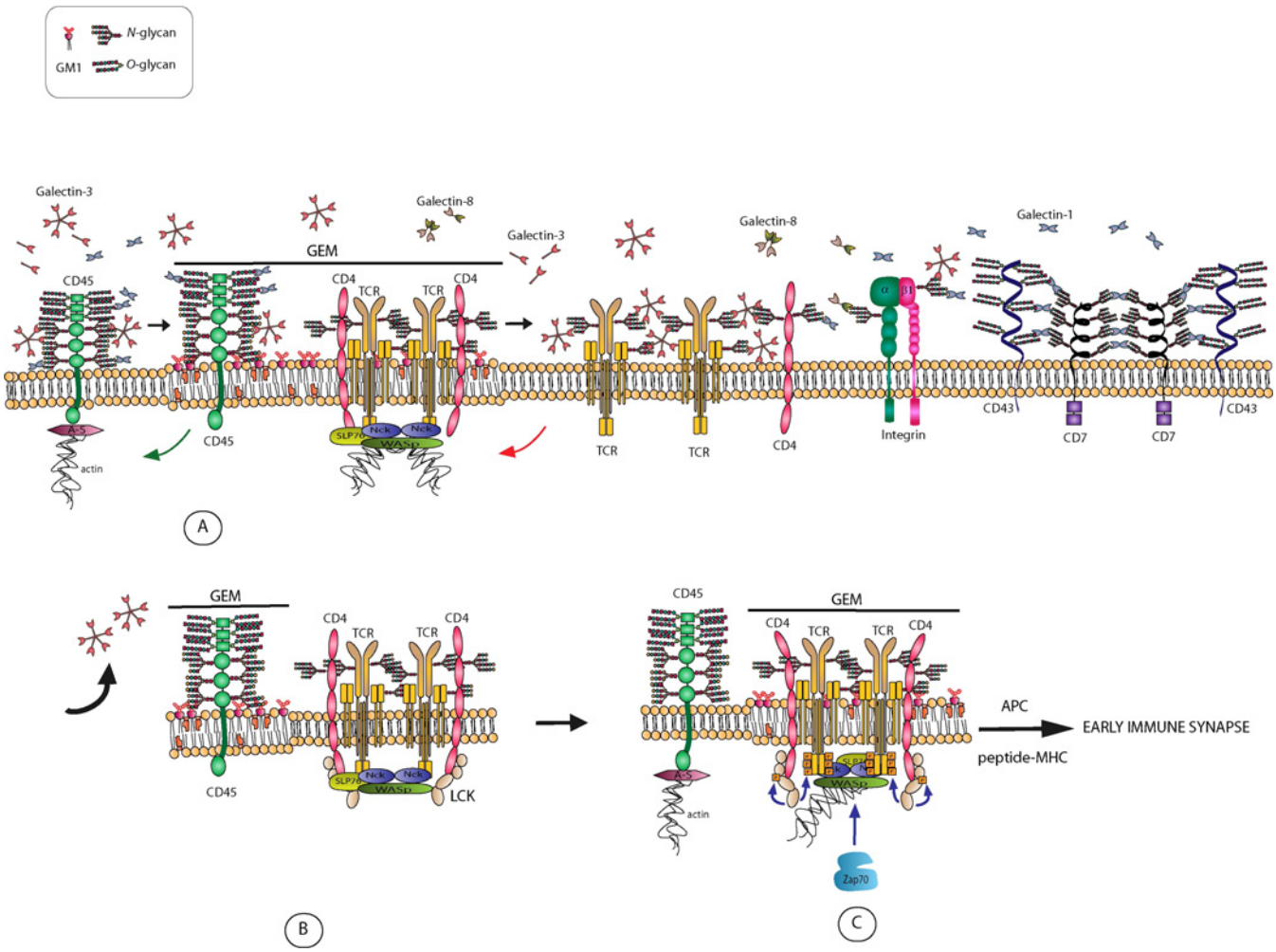

Figure 2 Galectin–glycan complexes regulate T cell functions and oppose to F-actin effects

(**A**) Different galectins cross-link several glycosylated receptors including TCR, CD45, CD4, CD43, CD7, integrins, GSLs (as GM1), and other glycoconjugates from the plasma membrane in resting T cells. Cytoplasmic domains of certain molecules interact with the actin cytoskeleton via adaptor molecules: *i.e.* CD45 interaction with F-actin via A-S (ankyrin-spectrin) promotes CD45 removal from the ganglioside GM1-enriched microdomains (GEMs) (green arrow). F-actin interaction with TCR -via Nck, SLP-76, and WASp- promotes TCR/CD4 partition to GEMs (red arrow). After galectin–glycan complex disruption (**B**), TCR clustering occurs, and Nck/WASp/SLP76/CD4 and Lck recruitment to TCR takes place. Then, (**C**) F-actin interaction with the TCR, via Nck, SLP-76, and WASp, promotes TCR/CD4 incorporation to GEMs. Moreover, F-actin-dependent partition of CD45 outside GEMs is also induced. Lck autophosphorylation at activating Tyr^{394} and its Lck-dependent targeting of Zap-70 to GEMs are triggered. Finally, the early immune synapse occurs when the antigen-presenting cell (APC) encounters naïve T cells. Adapted and modified from [89], with copyright permission from *J. Biol. Chem*.

CD45 is variable, with different isoforms expressed on different lymphocyte subsets at distinct developmental stages, which can include additional domains, termed A, B and C (encoded by exons 4, 5 and 6). The A, B and C domains all contain numerous serine and threonine residues, so that CD45 isoforms including these domains bear additional *O*-glycans, which can vary in number significantly among different isoforms [75]. Thymocytes and mature T-cells express the low-molecular-mass isoforms, typically CD45R0 (no additional domains), CD45RA and CD45RB. Thus, T-cells have relatively fewer *O*-glycans compared with B-cells, which express full-length CD45RABC with the complete repertoire of *O*-glycans [74]. CD45 also bears *N*-glycans, most of which are found on the membrane-proximal region of the molecule, which is common to all CD45 isoforms (Figure 2). When analysing glycophenotypes, immature cortical thymocytes were found to express abundant asialo-core-1 *O*-glycans that can bind the plant lectin PNA (peanut agglutinin), whereas expression of α2,3-sialyltransferase 1 (ST3Gal1), responsible for creating the sialic acid-α2,3Galβ1,3GalNAc sequence, is up-regulated in mature medullary thymocytes, which therefore display low PNA-binding activity. Immature cortical thymocytes bear core 2 *O*-glycans, created by the C2GnT1 glycosyltransferase (Figure 1), whose expression is reduced in mature medullary thymocytes [76]. Moreover, higher binding of SNA (*Sambucus nigra* agglutinin) (which binds α2,6-linked sialic acid primarily on *N*-glycans) was found in mature rather than immature thymocytes, indicating increased decoration of glycans with α2,6 sialic acid linkages on these cells [77]. Deletion of either ST3Gal1 or α2,6-sialyltransferase 1 (ST6Gal1), both of which preferentially regulate glycan structures on mature thymocytes, results in aberrant thymocyte development [76,78]. In fact, the most immature T-cells express neither CD4 nor CD8 ($CD4^-$ $CD8^-$), whereas immature cells that are undergoing TCR (T-cell receptor) rearrangement express both CD4 and CD8 ($CD4^+$ $CD8^+$), and mature cells, which express a functional TCR, carry either CD4 or CD8. PNA profiles showed that

$CD4^+$ $CD8^+$ cells are highly susceptible to Gal-1-induced cell death concomitant with positive selection of these cells, whereas $CD4^-$ $CD8^-$ and $CD4^+$ or $CD8^+$ thymocytes, which have reduced levels of asialo-core-1 *O*-glycans, are resistant to Gal-1-induced death. As mentioned above, several plant lectins have been used to determine cell-surface glycophenotypes by different authors, especially in T-cells, i.e. PNA, SNA and MAL II (*Maackia amurensis* lectin II), which binds α2,3-linked sialic acid primarily on *N*-glycans [77]. It has been found that peripheral mature $CD4^+$-Th2 cells, which are resistant to Gal-1, express ST6Gal1 and bind SNA with great avidity, indicating that addition of α2,6-linked sialic acid protects cells from Gal-1-induced death [34]. These results are in accordance with pioneering structural studies on Gal-1, suggesting that α2,6-linked sialic acid distorts the binding pocket of the lectin and prevents binding of LacNAc [22]. Glycosylation of CD45 is highly regulated in T-cells and has been studied in detail. Cells that express CD45, but not core 2 *O*-glycans, are not susceptible to Gal-1-induced death, whereas reinforced expression of C2GnT1 adds core 2 *O*-glycan structures to CD45 and renders cells susceptible to Gal-1 [65]. Moreover, Earl et al. [77] showed that Gal-1 signalling through CD45 is regulated by CD45 isoform expression, core 2 *O*-glycan formation and the balance of *N*-glycan sialylation. Addition of core 2 *O*-glycans by transfection with C2GnT1 in $CD45RB^+$ BW5147 thymoma cells resulted in increased Gal-1 binding to the cell surface and decreased tyrosine phosphatase activity. C2GnT1 was also transfected in the $T200^-$ cell line, a derivative of BW5147 cells that lacks CD45, and the Rev1.1 cell line, a derivative of $T200^-$ cells that bears a truncated form of CD45 expressing only its transmembrane and extracellular domains: the presence or absence of core 2 *O*-glycans had no effect on the susceptibility of either cells to Gal-1-induced cell death, demonstrating that the primary effect of core 2 *O*-glycan modification of CD45 is to allow Gal-1 regulation of CD45 tyrosine phosphatase activity. To determine whether an increase in total core 1 (low-affinity Gal-1 ligand) *O*-glycans could compensate for the lack of core 2 (high-affinity Gal-1 ligand) *O*-glycans, full-length murine CD45RABC was transfected into $T200^-$ cells with and without C2GnT1. The authors found no significant differences in the susceptibility to Gal-1-triggered death between cells co-transfected with CD45RABC and C2GnT1 and cells transfected with CD45RABC alone. Furthermore, $T200^-$ cells transfected with CD45RABC, but not with C2GnT1, showed a 20% reduction in phosphatase activity after Gal-1 treatment, showing that the greater abundance of low-affinity core 1 *O*-glycan ligands present on CD45RABC compared with CD45RB is sufficient to allow Gal-1 binding and signalling, even in the absence of core 2 *O*-glycan elongation. With regard to *N*-glycans in CD45, the binding efficiency of Gal-1 to bi-antennary *N*-glycans with two α2,3-, one α2,3- and one α2,6-, or two α2,6-linked, sialic acids was evaluated in glycan arrays, showing that lectin binding was dramatically reduced when both branches were terminated with α2,6-linked sialic acid, whereas the greatest binding was observed for the bi-antennary glycan with only α2,3-linked sialic acids. When α2,3-sialyltransferase 3 (ST3Gal3) was transfected in PhaR2.1 cells, Gal-1 binding was increased relative to parental PhaR2.1 cells, whereas it was decreased when ST6Gal1 was up-regulated. Moreover, ST3Gal3-transfected cells had a similar reduction in tyrosine phosphatase activity and a 2-fold increase in susceptibility to Gal-1-induced T-cell death compared with parental cells. In summary, the decoration of *O*-glycans with core 2 branches and *N*-glycans with terminal α2,6-linked sialic acid are tightly regulated during T-cell development and activation, so that both glycosylation events can alter the threshold for Gal-1 binding and signalling [77].

On T-cells, Gal-1 binding induces a dramatic redistribution of CD45 and CD43 on the surface. Before Gal-1 binding to MOLT-4 T-cell lines, a uniform distribution of CD45 and CD43, with occasional co-localization was observed, but, after lectin treatment, CD45 localized into one or two large islands on the cell surface, excluding CD43. Thus, the binding of Gal-1 to T-cells induced redistribution and segregation of CD45 and CD43. Because CD7 also bound Gal-1, its cell-surface distribution was also analysed: before Gal-1 treatment, CD45 and CD7 were uniformly distributed over the cell surface, with some regions of co-localization, whereas Gal-1 treatment produced both CD45 segregation into one or two large islands per cell and CD7 aggregation into multiple small clusters that segregated away from CD45. This pattern was identical with that observed for CD45 and CD43. Before Gal-1 treatment, CD7 co-localized with CD43, and, after Gal-1 treatment, CD43 and CD7 remained associated and moved into larger aggregates, indicating that CD43 and CD7 may act as a complex during the delivery of the Gal-1 death signal (Figure 2). Moreover, cell-surface localization of CD45 and CD3 was also studied by confocal microscopy. Uniform distribution of CD45 and CD3 on both immature $CD3^{low}$ and mature $CD3^{high}$ human thymocytes was detected before treatment, yet exposure to Gal-1 resulted in CD45 and CD3 co-localization into one island on $CD3^{low}$, whereas $CD3^{high}$ cells remained unaltered [66]. As CD3/CD45 segregation was not observed on cells that were refractory to Gal-1, such as mature thymocytes [67], the spatial redistribution of glycosylated receptors into specific microdomains seems to be a requirement for induction of Gal-1-induced T-cell death [66].

He and Baum [79] reported that Gal-1 inhibition of T-cell trafficking and migration was associated with altered T-cell distribution of CD43. In fact, localization of CD43 on the cell surface substantially differed between migrating and stationary T-cells, as it was localized to the uropod of migrating T-cells, determining migrating T-cell polarity [80,81]. When CD43 localization on migrating T-cells was evaluated in the presence or absence of Gal-1, cells attaching to control Matrigel displayed uniform cell-surface distribution of CD43, whereas cells spread on Gal-1-coated Matrigel showed CD43 clusters into large membrane patches, mainly at the T-cell/Matrigel interface. Thus Gal-1–CD43 interaction may prevent translocation of CD43 to the uropod or affect actin reorganization essential for cell migration [79].

In addition, clusters of CD71 have been reported in Gal-3-treated T-cells. In fact, several membrane glycoproteins that interact with Gal-3 were identified, such as Mac-2-binding protein, CD29 (β1-integrin), CD98, CD43, CD45, CD71 and the TCRβ chain. However, CD45, but not CD29 nor CD43, were found to contribute to T-cell death induced by extracellular (but not intracellular) Gal-3 [82]. Strikingly, clusters of CD71 were detected in Gal-3-treated T-cells, an effect which correlated with annexin V staining on membrane blebs, suggesting that CD71 redistribution is involved in Gal-3-mediated cell death [83].

In Th2 cells, Bi et al. [84] demonstrated that Gal-9 binds to PDI (protein disulfide-isomerase), an oxidoreductase enzyme, increasing its retention at the cell surface and leading to increased surface thiols [84]. PDI regulates the function of several integrins by reducing disulfide bonds to convert them into a high-affinity conformation [85]. Three proteins from Th2 cells, which were demonstrated to bind Gal-9 in a glycan-dependent manner, are members of the PDI family. Increased surface retention of PDI by Gal-9 was inhibited by lactose, and mediated by PDI *O*-glycans as it was abolished by inhibitors of *O*-glycan elongation (Figure 2). Gal-1 and Gal-3 had no effect on the abundance of cell-surface PDI, highlighting the specificity of Gal function. Moreover, Gal-9

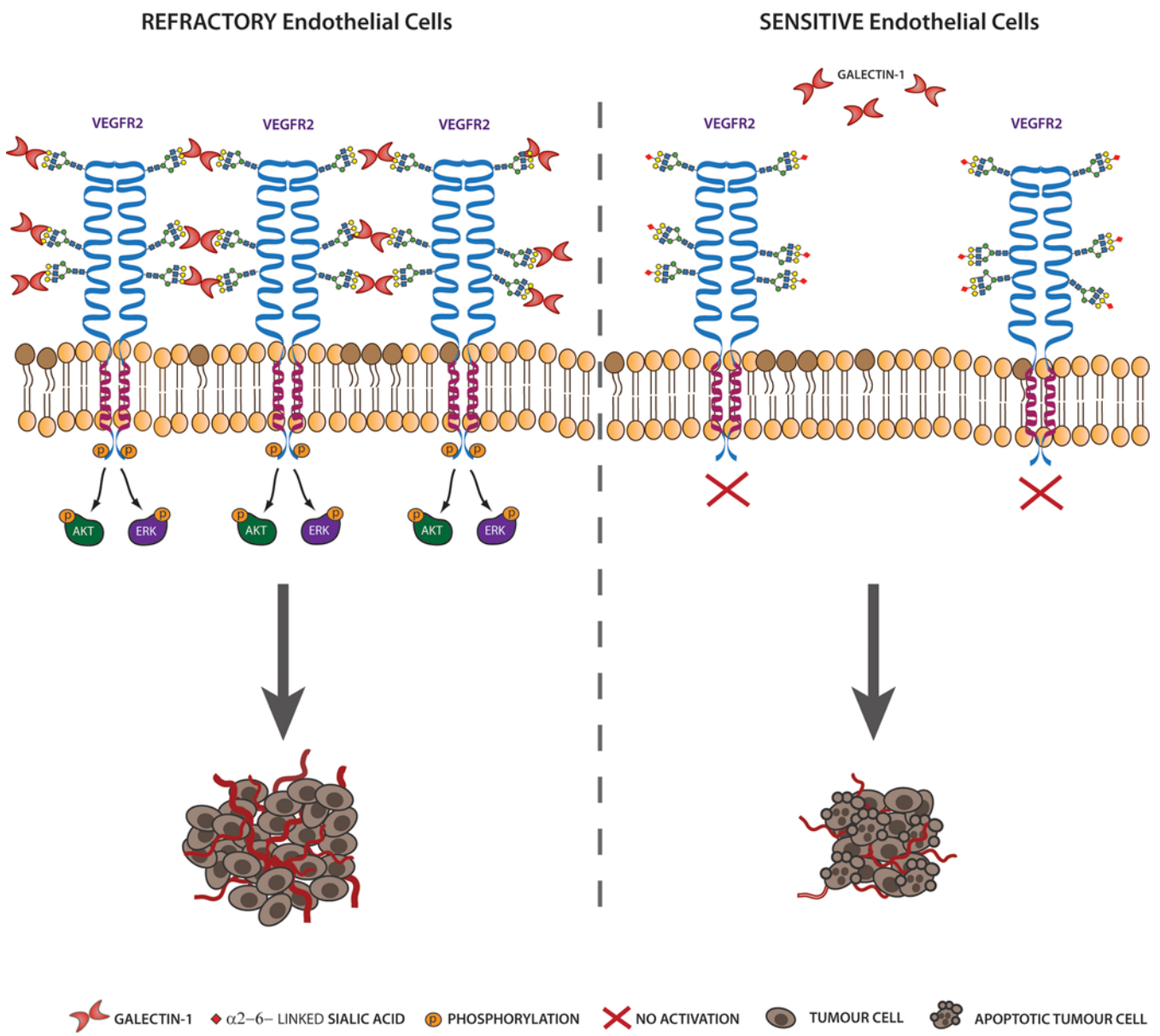

Figure 3 The interaction of Gal-1 with VEGFR2 functionally mimics VEGF-A signalling

Gal-1 interacts with complex *N*-glycans on VEGFR2 and enables receptor segregation and retention at the EC surface and promoting phosphorylation of downstream signalling (ERK1/2 and Akt) pathways. Tumour progression is accompanied by the generation of new blood vessels which, along with the activation of invasive pathways and immune escape mechanisms, enables tumour growth and metastasis. Anti-angiogenic therapies have emerged as a promising therapeutic strategy; however, their success is limited by innate and adaptive resistance mechanisms. Gal-1 promotes VEGF-like signalling and activates the VEGFR2 pathway even in the absence of VEGF, thus preserving angiogenesis in VEGF-targeted therapies. In response to VEGF blockade, anti-VEGF refractory tumours secrete higher amounts of Gal-1 and their ECs display increased β1,6-*N*-glycan branching, augmented poly-LacNAc extension and lower α2,6-sialylation, facilitating Gal-1 signalling, compensatory angiogenesis and tumour growth. Blood vessels associated with anti-VEGF-sensitive tumours display higher amounts of α2,6-linked sialic acid, which prevents Gal-1–VEGFR2 interactions, showing that the *N*-glycosylation status of tumour-associated vessels defines tumour sensitivity to anti-VEGF therapy.

enhanced T-cell migration across the matrix through binding to cell-surface PDI, thus promoting its retention, association with β3 integrin and increased disulfide reductase activity [84].

The TCR has seven *N*-glycans [86,87] and binds Gal-3 via GlcNAc-branched *N*-glycans, an interaction that was disrupted by either Mgat5 deficiency or by lactose [88]. TCR clustering was significantly greater in T-cells isolated from *Mgat5*$^{-/-}$ mice compared with those from *Mgat5*$^{+/+}$ mice, demonstrating that Mgat5 deficiency lowers T-cell activation thresholds. Moreover, pre-incubation with lactose in *ex vivo* isolated wild-type T-cells increased TCR clustering induced by anti-CD3ε antibody-coated beads, an effect which phenocopied the behaviour of untreated *Mgat5*$^{-/-}$ T-cells. Mgat5 deficiency increased the number of TCRs recruited to the antigen-presenting surface, thereby reducing the requirement for CD28 co-receptor engagement, which may lead to T-cell activation in the absence of CD28 co-signalling, rescue of anergy and loss of immune tolerance. Thus multivalent cell-surface Gal–glycoprotein interactions limit TCR clustering in response to agonist ligands, the avidity of which is dependent on Mgat5-modified *N*-glycans, restricting TCR recruitment to the site of antigen presentation [88]. Likewise, the Gal-glycan complex was shown to particularly cross-link TCR

and CD45 on resting T-cells, and to bind Gals in proportion to the number of N-X-S/T sites and the degree of GlcNAc branching within *N*-glycans [89]. CD45 can be modified by Mgat5, producing β1,6GlcNAc-branched *N*-glycans [90] that bind to endogenous Gal-3 at the cell surface, an interaction that was abolished by Mgat5 deficiency or by incubation with lactose. Co-localization studies of TCR, CD45 and CD4 with GEMs (ganglioside GM1-enriched microdomains) were assessed in resting mouse T-cells, demonstrating that disruption of the Gal–glycan lattice through Mgat5 deficiency or by co-incubation of wild-type cells with lactose significantly reduced co-localization of CD45 with GEMs (Figure 2). Moreover, Mgat5 deficiency or lactose treatment significantly enhanced the co-localization of TCR and CD4 to GEMs in resting T-cells. Therefore, in T-cells, the Gal–glycan complex promotes partition of CD45 within GEMs and TCR/CD4 outside GEMs (Figure 2). In this regard, activation of the Lck (leucocyte-specific protein tyrosine kinase) via autophosphorylation at Tyr^{394} is required for phosphorylating ZAP-70 (ζ-chain-associated protein kinase of 70 kDa), which in turn phosphorylates LAT (linker of activated T-cells), inducing agonist-induced TCR signalling and T-cell activation. CD45 exerts a fine control of Lck, as shown by the following evidence: (i) negative regulation of Lck-Tyr^{394} and TCR signalling by the Gal-glycan lattice was observed in the presence, but not in the absence, of CD45; (ii) partition of CD45 to GEMs after F-actin (filamentous actin) depolymerization is associated with reduced Lck-Tyr^{394} phosphorylation in the absence, but not in the presence, of a selective CD45 phosphatase inhibitor; and (iii) forced localization of CD45 to the early immune synapse inhibits Lck-Tyr^{394}, ZAP-70 and LAT phosphorylation. In conclusion, within microdomains, maintenance of Lck-Tyr^{394} after autophosphorylation is negatively regulated by CD45 [89]. Actin reorganization is required for clustering of the TCR and GEMs at the immune synapse [91], and blocking F-actin polymerization with latrunculin-A in resting mouse $Mgat5^{+/+}$ T-cells reduces TCR/CD4 and increases CD45 localization within GEMs, an effect that is abolished upon Gal–glycan lattice disruption. The cytoplasmic domain of CD45 binds ankyrin, an association proposed to maintain CD45 outside GEMs via interaction of this membrane-associated adaptor protein and its partners (i.e. ankyrin/spectrin) with actin microfilaments [92]. Disruption of F-actin with latrunculin-A increases CD45 localization to GEMs and reduces Lck-Tyr^{394} phosphorylation in resting $Mgat5^{+/+}$ T-cells, whereas co-incubation of lactose with latrunculin-A normalizes CD45 content in GEMs and reverses latrunculin-A-induced Lck-Tyr^{394} hypophosphorylation. These data indicate that F-actin polymerization mediates the redistribution of TCR/CD4 and CD45 after disruption of the Gal–glycan complex [89]. Moreover, a critical regulator of actin reorganization in T-cells is the WASp (Wiskott–Aldrich syndrome protein), an actin nucleation-promoting factor, which binds Arp2/3 (actin-related proteins 2/3) to initiate F-actin polymerization [93]. Nck (non-catalytic region of tyrosine kinase) adaptor protein binds WASp, which recruits actin to the TCR during T-cell activation. Nck binds directly to the cytoplasmic tail of CD3ε upon a conformational change induced by agonist-mediated clustering of two TCR complexes [94,95]. Importantly, in the absence of TCR agonists, the Gal–glycan complex actively prevents clustering of two TCR complexes (Figure 2) and the recruitment of Nck/WASp to the TCR. Disrupting Gal–glycan complexes in resting Jurkat T-cell lines favoured Nck binding to the TCR complex and WASp association with Nck. Thus disruption of Gal–glycan complexes results in: (i) Nck/WASp/SLP76 (Src homology 2 domain-containing leucocyte protein of 76 kDa)/CD4 recruitment to the TCR; (ii) F-actin-dependent partition of CD45 outside GEMs concurrent with and independent of Nck/WASp/SLP76/F-actin-mediated TCR/CD4-Lck partition to GEMs; (iii) Lck autophosphorylation at activating Tyr^{394} and Lck-dependent targeting of Zap-70 to GEMs; and (iv) encounter with the antigen-presenting cell, coalescence of TCR-enriched and CD45-deficient GEMs during early immune responses (Figure 2). Thus a relevant mechanism for controlling membrane microdomain structure and function was described by competition of Gal–glycan complexes with F-actin, which occurs extracellularly through Gal binding to GlcNAc-branched *N*-glycans attached to extracellular domains of Gal receptors and intracellularly through the interaction of receptor cytoplasmic domains with adaptor proteins/polymerized F-actin [89].

Gal–glycan complexes also promote surface retention of CTLA-4 (cytotoxic T-lymphocyte antigen-4) on T-cell blasts, a phenotype that inhibits TCR signalling and induces growth arrest [96]. After some rounds of division, activated T-cells induce CTLA-4 expression on the cell surface and growth arrest in G_1-phase; in fact, surface expression of CTLA-4 (bearing two *N*-glycosylation sites) is induced 3–5 days after TCR signalling [97]. As mentioned above, interruption of β1,6GlcNAc-branching in naive T-cells reduces activation thresholds by weakening Gal–glycan interactions, enhancing TCR clustering and signalling at sites of immune synapse [88]. Lau et al. [96] showed that surface expression for CTLA-4 was lower on $Mgat5^{-/-}$ and $Mgat5^{+/-}$ than on $Mgat5^{+/+}$ T-cells following anti-CD3 antibody stimulation, but reached comparable levels following strong co-stimulation with anti-CD3 and anti-CD28 antibodies. Swainsonine treatment blocked GlcNAc-induced surface expression of CTLA-4. Moreover, TCR hypersensitivity of $Mgat5^{-/-}$ T-cells was associated with increased intracellular CTLA-4 following activation, consistent with a specific defect in cell-surface retention. Analysis of gene expression profiles following antigen-driven immune synapse formation revealed an increase in *Il2* and *Mgat5* gene transcription, which generated positive feedback to glucose metabolism, hexosamine flux, *N*-glycan branching, lattice strength and, finally, CTLA-4 surface retention. In summary, multimeric Gal–glycan interactions negatively regulate T-cell responses early by inhibiting TCR/CD28 signalling in naive T-cells and later by promoting retention of CTLA-4 on the surface of T-cell blasts [96].

Within the bone marrow, Gal-1 synthesized by stromal cells has been identified as a ligand for the pre-BCR (B-cell receptor), specifically for the surrogate light chain, in pre-B-cells [98]. B-cell differentiation is a highly regulated process, which involves the transition from pro-B- to pre-B- and immature B-cells. At the pre-BII-cell stage, newly generated IgH chains are probed for their ability to interact with surrogate light chains and form a pre-BCR that will be expressed at the cell surface [99]. The pre-BCR is composed of two Igμ chains, two surrogate light chains (composed of the λ5 and VpreB invariant proteins) and the CD79a/b signalling molecules [100]. Gal-1 secreted by stromal cells is captured by the pre-BCR and counter-receptors on B-cells (*cis*-counter-receptors) and on stromal cells (*trans*-counter-receptors). Gal-1 binding to pre-B-cells leads to formation of pre-BCR–Gal-1 complexes, polarized at the contact zone between pre-B- and stromal cells, which trigger pre-BCR signalling. Therefore, Gal-1 has been considered as a supramolecular organizer of a tridimensional lattice that clusters together glycoconjugate receptors and the pre-BCR [98]. NMR studies provided the first molecular model of a pre-BCR–ligand interaction that promoted pre-BCR clustering, identifying a conserved Gal-1-interacting peptide region within the full-length λ5 (called $\lambda5\text{-}UR^{22–45}$) of BCR, which involves hydrophobic protein–protein interactions; this Gal-1–pre-BCR association is

the first example of a Gal-1–unglycosylated protein interaction in the extracellular compartment. However, the lactose-binding activity of Gal-1 in the absence or in the presence of the λ5-UR$^{22–45}$ peptide showed a 4-fold reduction when Gal-1 was bound to λ5-UR$^{22–45}$, as evaluated by NMR, suggesting that the peptide binds to Gal-1 on a surface close to the CRD and induces changes in Gal-1–glycan interactions [101]. Similarly, when the carbohydrate-binding specificity of Gal-1 free or bound to the λ5-UR peptide was analysed in glycan array experiments and isothermal titration calorimetry, binding to LacNAc was not altered whereas binding to higher elongated and branched poly-LacNAc-containing carbohydrates was markedly decreased. These data suggest that λ5-UR binding to the lectin differentially modulates Gal-1-binding activity, and thus Gal-1–pre-BCR interaction induces local conformational changes in the Gal-1 carbohydrate-binding site, generating a reduction in Gal-1–glycan affinity. Therefore, unglycosylated protein binding to Gal-1 could also represent a key regulator of Gal-1–glycan interactions in the extracellular compartment [102]. When pre-B-cells were co-cultured with stromal cells, pre-BCR and Gal-1 clustering was observed at the pre-B-cell–stromal cell contact site using confocal microscopy, and the addition of the full-length λ5-UR inhibited the pre-BCR relocalization process by 25 %. In this model, constitutive pre-BCR activation is assisted by stromal-derived Gal-1, strengthening pre-BCR oligomerization and leading to synapse formation and pre-BCR activation. The requirement of Gal-1 could be linked to the level of pre-BCR surface expression. Thus, under low pre-BCR surface expression (as is mainly the case for normal pre-BII-cells *in vivo*), Gal-1-induced signalling would be required to amplify pre-BCR functions, whereas with high BCR expression, Gal-1 could not be necessary for pre-BCR oligomerization [101]. Clustering of pre-B-cell integrins also induces Gal-1-dependent pre-BCR relocalization and activation, creating Gal–glycan complexes at the interface between pre-B-cells and stromal cells. Gal-1 was demonstrated to bind directly to $\alpha5$, $\beta1$ and $\beta7$ integrins from Nalm6 pre-B-cells and to $\alpha5\beta1$ integrin from MS5.1 stromal cells. Both integrins and integrin ligands are polarized together with Gal-1 and the pre-BCR into the pre-B-cell–stromal cell synapse. To identify whether integrins, major Gal-1-glycosylated counter-receptors, were involved in the synapse formation, confocal microscopy analysis of stromal and pre-B-cell co-cultures was performed, revealing that integrin staining co-localized with those of the pre-BCR and Gal-1. $\alpha4\beta1$ and $\alpha5\beta1$ integrins on pre-B-cells and the ADAM15 (a disintegrin and metalloprotease 15) integrin ligand on stromal cells co-localized with the pre-BCR and Gal-1 at the contact area between pre-B- and stromal cells. Moreover, lactose blocked pre-BCR relocalization, whereas that of $\alpha4$ and $\alpha5$ integrins was not altered. In conclusion, pre-B-cell integrins and their stromal cell ligands (ADAM15/FN), together with the pre-BCR and Gal-1, form an homogeneous Gal-1–glycan complex at the contact area between pre-B-cells and stromal cells, promoting activation of the pre-BCR [103].

In human monocyte-derived DCs, Gal-1 induces membrane reorganization, cell activation and migration. When Gal-1 receptors from monocyte-derived DCs were analysed, the lectin specifically bound to CD43 and CD45 [104]. Moreover, after Gal-1 binding, confocal microscopy allowed visualization that, in contrast with the phenotype seen on T-cells where CD43 and CD45 are clustered but segregated into different domains [66], both CD43 and CD45 co-clustered in an unipolar fashion on the DC cell membrane, and the pre-incubation with lactose inhibited their redistribution. However, Gal-1 did not cause global membrane reorganization, as was evident from a lack of redistribution of CCR5 (CC chemokine receptor 5), a highly expressed cell-surface protein on DCs that is not known to bind Gal-1. Gal-1, but not LPS (lipopolysaccharide) or the buffer control, specifically induced calcium flux, which was dose-dependent and abrogated by lactose. Before Gal-1 treatment, CD43 and CD45 and basal levels of Syk (spleen tyrosine kinase) phosphorylation were uniformly distributed around the cell membrane, as shown by confocal microscopy; however, with lectin treatment, pSyk co-localized with the CD43 and CD45 co-cluster, an effect which was prevented by lactose, suggesting that CD43–CD45–pSyk clusters may be critical for Gal-1 modulation of DC function, including its pro-migratory and tolerogenic activity [104]. Through binding and cross-linking CD43, Gal-1 triggers an IL-27-mediated tolerogenic phenotype on DCs, which in turn amplifies Treg cell responses via induction of IL-10, thus contributing to tumour-immune escape and the resolution of autoimmune inflammation [60].

Remarkably, Gal-3 binding also forms patches or clusters on the surface of unprimed neutrophils [25,105]. Elegant experiments using FRET analysis with fluorescently labelled Gal-3 at the C-terminus demonstrated the oligomerization of Gal molecules on the surfaces of these cells [25]. Internal fluorescence transfer from Gal-3–Alexa Fluor 488 (donor) to Gal-3–Alexa Fluor 555 (acceptor) was observed, and firm lattices were detected in both unprimed and primed neutrophils at 4 °C, whereas at 37 °C lattices were relatively stable only in unprimed neutrophils. As Gal-3 interaction with primed neutrophils leads to lectin deactivation through cleavage by elastase, Gal-3–Alexa Fluor 488 and Gal-3–Alexa Fluor 555 were incubated with elastase, and the resulting truncated Gal-3 was incubated with unprimed neutrophils. Binding of truncated Gal-3 to neutrophils was lower than binding of full-length Gal-3 as revealed by a diminished FRET signal, thus substantiating the importance of the N-terminal domain of this lectin in the oligomerization process. A FRET signal could also be detected when Gal-3–Alexa Fluor 488 and Gal-3–Alexa Fluor 555 were incubated with neutrophils in the presence of an EC (endothelial cell) layer, where the importance of oligomerization was also demonstrated [25].

Within the microglial compartment, Gal-1–glycan interactions are also essential in tempering microglia activation by specifically retaining CD45 on the cell surface. We have identified Gal-1 as a pivotal regulator of microglia functions by targeting the activation of p38 MAPK (mitogen-activated protein kinase)-, CREB (cAMP-response-element-binding)-, and NF-κB (nuclear factor-κB)-dependent signalling pathways and suppressing downstream pro-inflammatory mediators such as iNOS (inducible nitric oxide synthase), TNFα (tumour necrosis factor α) and CCL2 (chemokine ligand 2). In fact, the lack of Gal-1 favours classical microglia activation, polarization towards an M1-type profile, CNS (central nervous system) inflammation and neurodegeneration. Moreover, we found that astrocytes control microglia activation and CNS inflammation via Gal-1–*O*-glycan interactions. Gal-1 binds to isolated primary microglia in a dose- and saccharide-dependent fashion, and its binding is markedly increased when microglia are polarized towards an M1 phenotype. In contrast, Gal-1 binding to M2-polarized microglia is substantially decreased compared with unstimulated microglia. Assessment of the glycophenotype of these cells revealed augmented unsialylated core 1 *O*-glycans in M1-polarized [LPS- or IFN-γ (interferon γ)-treated], microglia compared with M2-polarized (IL-4-treated) microglia. Increased PNA reactivity indicated increased availability of glycan structures required for elongation of core 2 *O*-glycans through the action of the enzyme C2GnT1 (Figure 1), which, as mentioned above, favours LacNAc incorporation and Gal binding [106]. In addition, M1-type microglia showed a higher capacity to bind L-PHA

(leucoagglutinin from *Phaseolus vulgaris*), suggesting increased β1,6-branching of complex *N*-glycans that are generated by the enzyme Mgat5 [107], whereas M2-type microglia showed a trend towards higher binding of SNA consistent with the ability of α2,6-linked sialic acid to interfere with Gal-1 binding [106]. CD45 has been shown to negatively regulate M1 microglia activation, leading to the promotion of an M2 phenotype [108]. Co-immunoprecipitation experiments demonstrated that Gal-1 and CD45 are associated in microglia cells treated with Gal-1. Moreover, exogenously added Gal-1 co-localized with CD45 on LPS-stimulated M1 microglial cells, and showed considerably diminished co-localization of CD45 with EEA1 (early endosomal antigen 1) compared with cells treated with LPS alone, consistent with decreased internalization of CD45. Flow cytometric analysis of non-permeabilized cells confirmed a time-dependent retention of CD45 on the surface of LPS-stimulated M1 microglia exposed to Gal-1 compared with control cells, finally resulting in deactivation of these cells. Functionally, binding of Gal-1 to CD45 resulted in a time-dependent increase in phosphatase activity, an effect that could be eliminated in the presence of CD45-specific phosphatase inhibitors, indicating that increased phosphatase activity was completely attributed to CD45. The contribution of *N*- and *O*-glycans to this effect was evaluated by transfection of BV-2 microglia cells with siRNA for C2GnT1 and Mgat5: inhibition of core 2 *O*-glycan elongation through siRNA-mediated silencing of C2GnT1 almost completely eliminated CD45–Gal-1 interactions and Gal-1-induced CD45 phosphatase activity, whereas inhibition of complex-type *N*-glycan branching with Mgat5 siRNA had no substantial effect. Thus, *O*-glycan-dependent association of Gal-1 with CD45 promotes retention of this glycoprotein on the cell surface, augments its phosphatase activity and induces deactivation of microglial cells, contributing to prevention of inflammation-induced neurodegeneration [106].

Gal–glycan complexes at the surface of tumour cells

As mentioned above, β1,6GlcNAc-branched complex *N*-glycans play important roles in tumour development and progression because Mgat5-deficient mice showed suppression of tumour growth and metastasis. Thus, malignant transformation is associated with increased expression of β1,6GlcNAc-branched *N*-glycans, products of the Mgat5 glycosyltransferase, which is up-regulated in carcinoma cells, providing substrates for elongation of poly-LacNAc [43]. Partridge et al. [107] reported that expression of Mgat5 sensitizes mouse cells to multiple cytokines. Mammary epithelial tumour cells from transgenic mice carrying the PyMT oncogene were isolated from animals with $Mgat5^{+/+}$ or $Mgat5^{-/-}$ backgrounds and their responses to growth factors were compared. The authors found that $Mgat5^{-/-}$ tumour cells were less responsive than $Mgat5^{+/+}$ cells to EGF (epidermal growth factor), IGF (insulin-like growth factor), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor)-2 and FBS, but transduction of cells with a retroviral vector encoding Mgat5 restored responsiveness of these cells. Moreover, given that EGFR (EGF receptor) in carcinoma cells carries ten to twelve N-X-S/T consensus sites for *N*-glycosylation [109], whereas TGF (transforming growth factor)-β receptors TβRI and TβRII have only one and three sites respectively [110], the importance of the number of *N*-glycosylation sites was evaluated. The authors found that $Mgat5^{-/-}$ cells displayed a 2–3-fold decrease in sensitivity to TGF-β compared with the 100-fold decrease in sensitivity to EGF in $Mgat5^{+/+}$ control cells, suggesting the influence of the number of *N*-glycans per receptor in sensitivity to Gal–glycan complexes. Similar results were observed for other growth factor receptors with multiple *N*-glycosylation sites such as PDGF, IGF-1 and FGF receptors. By chemical cross-linking assays, EGFR was shown to be associated with Gal-3 on the surface of $Mgat5^{+/+}$ cells, whereas this interaction was greatly diminished in $Mgat5^{-/-}$ cells. Pre-treatment with lactose also depleted surface Gal-3 on $Mgat5^{+/+}$ cells and diminished EGF-dependent activation of ERK1/2 (extracellular-signal-regulated kinase 1/2) MAPK. Thus, surface distribution of Gal-3 and its glycoprotein receptors are highly dependent on Mgat5 modification of receptor *N*-glycans. Moreover, $Mgat5^{-/-}$ cells displayed 4-fold greater co-localization of EGFR with EEA1 than $Mgat5^{+/+}$ cells, denoting increased internalization of EGFR in the absence of complex *N*-glycan branching. Pre-treatment of $Mgat5^{+/+}$ cells with lactose, but not sucrose, promoted receptor accumulation in early endosomes, mimicking the distribution observed in untreated $Mgat5^{-/-}$ cells. Thus, upon lattice disruption, the number of receptor molecules increased in early endosomes. Mgat5 deficiency could be chemically rescued by K^+ depletion and nystatin treatment, agents that inhibit endocytosis, as well as by genetic manipulation using a retroviral vector that restores Mgat5 expression. However, expression of a mutant Mgat5-L188R, which could not enter the Golgi, failed to rescue complex *N*-glycan branching and signalling. TβRII also associates with Gal-3 in an Mgat5-dependent manner, and this interaction could be blocked by lactose. TGF-β signalling in Mgat5-L188R mutant cells was restored by infection with the Mgat5 retroviral vector and by blocking endocytosis with the use of K^+ depletion and nystatin. Moreover, $Mgat5^{+/+}$ tumour cells displayed EMT (epithelial–mesenchymal transition) with loss of adhesion junctions, whereas $Mgat5^{-/-}$ cells retained an epithelial morphology. Hence, when $Mgat5^{+/+}$, $Mgat5^{-/-}$ and mutant cells were infected with Mgat5 retroviral vectors, EMT could be induced in $Mgat5^{-/-}$: $Mgat5^{+/+}$ and Mgat5-rescued Mgat5-L188R mutant cells displayed greater *in vitro* cell motility and a higher number of spontaneous *in vivo* lung metastases than $Mgat5^{-/-}$ cells, showing that EMT and the malignant phenotype are dependent on Mgat5 expression by tumour cells [107].

Gal-3 interactions with Mgat5-modified *N*-glycans on PyMT mammary carcinoma cells have also been shown to regulate FN fibrillogenesis [111], which involves the dissociation of $\alpha5\beta1$ integrin from FAs (focal adhesions) and its translocation to fibrillar adhesions [112]. Spreading of $Mgat5^{+/+}$ PyMT mammary tumour cells increased with FN density, whereas $Mgat5^{-/-}$ tumour cells lacked the ability to spread on FN. Treatment of $Mgat5^{+/+}$ cells with swainsonine (an inhibitor of Golgi α-mannosidase II that blocks *N*-glycan GlcNAc branching and prevents processing by Mgat5) or with lactose, but not sucrose, reduces FN fibrillogenesis, suggesting that Gals and their high-affinity ligands β1,6GlcNAc-branched *N*-glycans are both required for this effect. Addition of recombinant Gal-3 to the cell medium stimulated FN matrix remodelling and cell motility of $Mgat5^{+/+}$, but not $Mgat5^{-/-}$, cells plated on a FN substrate in a dose-dependent manner. Gal-3 binding to Mgat5-modified *N*-glycans induced $\alpha5\beta1$ integrin activation in PyMT mammary tumour cells, and subsequent integrin-mediated activation of FAK (focal adhesion kinase) and PI3K (phosphoinositide 3-kinase), leading to increased FN fibrillogenesis and FN-dependent tumour cell spreading and motility [111].

Interestingly, the cellular transition between growth and arrest has been shown to be regulated at the metabolic level and associated with *N*-glycan number and branching [96]. Thus, metabolic supplementation of the hexosamine pathway was studied in $Mgat5^{+/+}$ and $Mgat5^{-/-}$ PyMT mammary epithelial tumour cells by the addition of GlcNAc. The authors found

reduced binding of Gal-3 to the surface of *Mgat5*$^{-/-}$ cells, which could be rescued by GlcNAc supplementation. Moreover, binding of EGF and TGF-β, as well as Gal-3 association with EGFR, in *Mgat5*$^{-/-}$ cells could also be rescued by GlcNAc. The extent of *N*-glycan branching doubled in *Mgat5*$^{-/-}$ cells supplemented with GlcNAc, suggesting that less-branched *N*-glycans in larger quantities are sufficient to restore Gal-3 binding and levels of surface receptors, whereas GlcNAc supplementation in *Mgat5*$^{+/+}$ cells only produced small increases in tri- and tetra-antennary *N*-glycans. Effects on *N*-glycan branching in response to hexosamine flux were analysed by MALDI–MS, showing an increase in the tri-antennary *N*-glycan fraction (the Golgi pathway end-product in *Mgat5*$^{-/-}$ cells) (Figure 1) in *Mgat5*$^{-/-}$ compared with *Mgat5*$^{+/+}$ cells. In fact, growth factor receptors, which usually stimulate cell proliferation, growth and oncogenesis, exhibit high numbers of *N*-glycans and high avidities for Gal–glycan complexes. Simulations where endocytosis rates are varied indicate that receptors with low numbers of *N*-glycans (i.e. one or two sites) are much more sensitive to cell-surface removal by endocytosis due to inherently lower avidities for the Gal–glycan complex than receptors with high numbers of *N*-glycans. Thus, a crucial conditional regulation of branching by nutrient flux through the hexosamine pathway was identified, which first stimulates growth via growth factor receptors that possess high numbers of *N*-glycans, and, when a higher flux is achieved, it induces arrest and differentiation through receptors with low numbers of *N*-glycans [96].

Interestingly, Boscher et al. [113] reported that Gal-3 accumulates at cell–cell junctions with N-cadherin (neural cadherin) and GM1, and contributes to the destabilization of cell–cell junctions. N-cadherin is a mesenchymal cadherin bearing seven *N*-glycosylation sites, which is overexpressed in many cancers and is associated with cancer cell migration, metastasis and FGF receptor signalling [114,115]. Incubation of *Mgat5*$^{+/+}$ PyMT mammary epithelial tumour cells with Gal-3 revealed intense cell-surface labelling and strong accumulation of Gal-3 at cell–cell junctions where it co-localizes with N-cadherin. Treatment with swainsonine or lactose inhibited Gal-3 recruitment to cell–cell junctions. Immunoprecipitation experiments in *Mgat5*$^{+/+}$ cells allowed the detection of endogenous Gal-3 in N-cadherin immunoprecipitates following cross-linking. The authors found that Gal-3 is recruited at cell–cell junctions by direct interaction with branched *N*-glycans on N-cadherin. The Gal–glycan complex and the dynamic properties of N-cadherin and GM1 were also evaluated in *Mgat5*$^{+/+}$ and *Mgat5*$^{-/-}$ cells at cell–cell junctions by FRAP (fluorescence recovery after photobleaching). Results showed that Gal-3 destabilizes cell–cell junctions and increases junctional mobility of N-cadherin and the GM1 ganglioside, suggesting that this lectin is a novel regulator of N-cadherin dynamics and cell–cell junction stability [113].

Gal-3 and Cav1 (caveolin-1) have been shown to regulate FAs, which mediate interaction between the ECM and cytoskeletal proteins in tumour cells via integrin aggregation [116]. Integrin binding to ECM and clustering of integrins induces autophosphorylation (Tyr^{397}) of FAK, the major kinase implicated in FA signalling [117]. Immunofluorescence and Western blot analysis revealed that FAK was mostly localized at the cytoplasm and its phosphorylation was reduced in *Mgat5*$^{-/-}$ cells compared with *Mgat5*$^{+/+}$ cells, suggesting that *Mgat5*$^{-/-}$ cells are deficient in FAs. FAK stabilization in FAs was shown to require Cav1-tyrosine phosphorylation, but in cells lacking the Mgat5-dependent Gal–glycan lattice, pCav1 is not sufficient to promote FAK stabilization. In support of the essential role of both Gal-3 and Cav1 in FA turnover, knockdown of either protein by siRNA was associated with reduced migration of *Mgat5*$^{+/+}$ cells. Similarly, when the Gal–glycan complex was disrupted with lactose, swainsonine or Gal-3 siRNA, Cav1-dependent stabilization of FAK was prevented. In summary, pCav1 and the Mgat5-dependent Gal-3-glycan complex stabilize FAK, paxillin and α5 integrin in FAs, thereby promoting FA turnover and favouring a migratory phenotype in tumour cells [116].

Gal–glycan complexes at the EC surface

Regulated glycosylation controls a variety of EC processes including Notch receptor signalling [118], EC survival [119], vascular permeability [120] and connection between blood and lymphatic vessels [121]. Changes in the cellular glycome can also affect vascular biology by displaying or masking ligands for endogenous lectins, which translate glycan-containing information into functional responses [53]. Expression of Gal-1 is regulated by hypoxia [122,123] and controls EC signalling [124], VEGFR (vascular endothelial growth factor receptor) trafficking [125] and tumour angiogenesis [126–129]. We showed that glycosylation-dependent interactions of Gal-1 with VEGFR2 [KDR (kinase insert domain-containing receptor)/Flk-1 (fetal liver kinase 1)] promoted segregation of this receptor into membrane microdomains and prolonged its cell-surface residency either in the presence or in the absence of the canonical ligand VEGF (vascular endothelial growth factor) [130].

Non-activated primary HUVECs (human umbilical vein endothelial cells) showed considerable expression of L-PHA-reactive Mgat5-modified *N*-glycans and poly-LacNAc ligands, which increased significantly following exposure to immunosuppressive cytokines (IL-10 or TGF-β_1) or to pro-angiogenic FGF-2. Notably, ECs exposed to pro-inflammatory Th1 (IFN-γ) or Th17 (IL-17) cytokines showed significantly lower L-PHA reactivity. Moreover, stimulation of ECs with FGF2 or a combination of IL-10 and TGF-β_1 led to a reduction in α2,6-sialic acid compared with resting, IL-17- or IFN-γ-treated ECs, suggesting that pro- or anti-inflammatory signals may either mask or unmask Gal-1-specific binding sites. Furthermore, exposure of HUVECs to FGF-2, IL-10 or TGF-β_1 resulted in a modest increase in PNA-reactive asialo-core-1 *O*-glycans, compared with cells exposed to IFN-γ or IL-17. Thus, immunosuppressive and pro-angiogenic stimuli favour a Gal-1-permissive glycophenotype on ECs, whereas pro-inflammatory signals tend to reduce expression of these glyco-epitopes. Accordingly, Gal-1 binding to ECs was almost completely abrogated by swainsonine, whereas benzyl-αGalNAc, an inhibitor of *O*-glycan elongation, was only partially inhibitory, suggesting the essential contribution of *N*-glycans to Gal-1 signalling. Likewise, a decrease in *N*-glycan branching through siRNA-mediated silencing of Mgat5 almost completely eliminated Gal-1 binding, whereas inhibition of core 2 *O*-glycan elongation through silencing of the C2GnT1 glycosyltransferase had no considerable effect. In fact, hypoxia increased the amounts of β1,6GlcNAc-branched *N*-glycans and poly-LacNAc structures, reduced α2,6-sialylation, and induced slight changes in asialo-core-1 *O*-glycans in comparison with normoxia. Moreover, relative abundance of neutral *N*-glycans and a decrease in tri- and tetra-sialylated *N*-glycans correlated with higher binding of Gal-1 to ECs cultured under hypoxic compared with normoxic conditions. Thus, immunosuppressive cytokines and hypoxic microenvironments induce ECs to express the entire repertoire of glycans that are critical for Gal-1 binding and angiogenesis, including increased β1,6-*N*-glycan branching, higher poly-LacNAc extension and lower α2,6-sialylation. Lactose, a decrease in *N*-glycan branching through

Mgat5 silencing or swainsonine treatment almost completely prevented EC proliferation, migration and tube formation induced by Gal-1, whereas silencing C2GnT1 had no effect. Pro-angiogenic effects of VEGF-A were maintained in both the absence and the presence of complex *N*- or *O*-glycan branching. Thus, unlike VEGF-A, Gal-1 delivers pro-angiogenic signals through a glycosylation-dependent pathway involving complex *N*-glycans. Moreover, VEGFR2 was the only RTK (receptor tyrosine kinase) tested that became phosphorylated following treatment of ECs with Gal-1, which in turn enhanced the phosphorylation of Akt and ERK1/2, recapitulating the phosphorylation pattern elicited by VEGF-A. Silencing VEGFR2 almost completely prevented Akt and ERK1/2 phosphorylation induced by either Gal-1 or VEGF-A and blocked Gal-1 induced migration and tube formation. These effects were confirmed by blocking experiments showing that inhibition of VEGFR2, but not VEGFR1, VEGFR3 or integrins, suppressed Gal-1 function. Silencing of Mgat5 selectively eliminated responsiveness to Gal-1, but it had no impact on VEGF-A signalling, whereas blockade of core 2 *O*-glycan elongation had no effect on Gal-1 or VEGF-A activity. Further experiments revealed that Gal-1 associated with VEGFR2 through *N*-glycosylation-dependent mechanisms. Thus Gal-1 directly co-opts the VEGFR2 pathway through binding to complex *N*-glycans. Using a series of human VEGFR2 mutants that are devoid of *N*-glycosylation sites in each of its seven Ig-like domains, we found that Gal-1 co-opts the VEGFR2 signalling pathway through binding to non-sialylated complex *N*-glycans on Ig3, Ig4 and Ig7 domains. Importantly, binding of Gal-1 resulted in *N*-glycan-dependent segregation of VEGFR2 to membrane patches, indicating rearrangement of signalling clusters on the EC surface (Figure 3). Moreover, flow cytometry of VEGFR2 in non-permeabilized ECs treated with VEGF-A, Gal-1 or VEGF-A plus Gal-1 showed higher surface retention of VEGFR2 and prevention of its internalization following exposure to this lectin. Similarly, confocal microscopy of VEGFR2 and EEA1 in permeabilized ECs treated or not with VEGF-A, Gal-1 or both demonstrated a reduced internalization index in ECs cultured in the presence of Gal-1 or Gal-1 plus VEGF-A compared with VEGF-A alone. *In vivo*, glycosylation-dependent interactions between Gal-1 and VEGFR2 resulted in sustained tumour angiogenesis and tumour growth. Lack of β1,6GlcNAc-branched *N*-glycans in ECs or silencing of tumour-derived Gal-1 converted refractory into anti-VEGF-sensitive tumours, whereas elimination of α2,6-linked sialic acid limited the efficacy of anti-VEGF treatment in sensitive tumours. Consequently, *in vivo* administration of a neutralizing anti-Gal-1 antibody promoted tumour growth inhibition and circumvented compensatory angiogenesis induced by VEGF blockade [130]. These data support the concept that glycosylation-dependent Gal-1–VEGFR2 interactions may compensate for the absence of VEGF in anti-VEGF-treated refractory tumours (Figure 3).

Gal-3 was also reported to retain VEGFR2 on HUVEC plasma membrane, whereas VEGFR2 interaction with VEGF-A induced rapid receptor internalization [131]. Previous studies revealed that VEGF-A-mediated angiogenesis is reduced *in vitro* by the addition of a dominant-negative Gal-3 or lactose; similarly, angiogenesis induced by VEGF-A was significantly reduced in Gal-3-deficient (*Lgals3*$^{-/-}$) mice [132]. VEGFR2 was later described as a Gal-3 ligand in HUVEC lysates incubated with a Gal-3 affinity column. Moreover, incubation of ECs with Gal-3 resulted in time-dependent phosphorylation of VEGFR2, which was increased with higher concentrations of the lectin, suggesting that exogenous Gal-3 could activate VEGFR2. When VEGFR2 internalization was evaluated after stimulation with VEGF-A, Mgat5- and Gal-3-knockdown cells displayed significantly greater intracellular co-localization of VEGFR2 and EEA1 compared with control cells, demonstrating increased endocytosis of the receptor. In fact, Gal-3 retained VEGFR2 on the plasma membrane in a carbohydrate-dependent manner [131]. In summary, Gal-3 binds to *N*-glycans on VEGFR2, facilitating its plasma membrane retention and phosphorylation; in addition, reduced *in vitro* angiogenesis in both Gal-3- and Mgat5-knockdown ECs and diminished *in vivo* corneal neovascularization in *Lgals3*$^{-/-}$ and *Mgat5*$^{-/-}$ mice were noticed [131]. Thus, by regulating VEGFR2 segregation, internalization and phosphorylation, Gal-3 contributes to VEGF-A-mediated angiogenesis and could therefore be a useful target for the development of antiangiogenic therapies.

Gal–glycan complexes at the surface of pancreatic β-cells

In pancreatic β-cells, surface expression of Glut-2 (glucose transporter 2) is critical for cell function, and Mgat4a has been shown to be implicated in its proper *N*-glycosylation [133]. Mgat4a and Mgat4b are catalytically redundant and initiate the synthesis of the β1,4GlcNAc branch on the core Manα1-3 arm (Figure 1). Mgat4a expression is prominent in pancreatic and gastrointestinal tissues, whereas Mgat4b is widely expressed in most tissues [133]. Ohtsubo et al. [134] engineered mice lacking *Mgat4a* gene function by producing a deletion in exon 7 of this gene, which abrogates the enzymatic activity. Mgat4a-deficient mice had impaired glucose transport and insulin secretion by β-cells, resulting in Type 2 diabetes. In wild-type pancreatic β-cells, it was demonstrated that Glut-2 associates with Gal-9 at the cell surface, an interaction that could be disrupted by LacNAc, but not sucrose, suggesting the involvement of the unique Glut-2 *N*-glycosylation site. Glut-2 in Mgat4a-deficient pancreatic β-cells showed diminished cell-surface expression and increased accumulation in early endosomes and lysosomes, as detected by co-localization with EEA1 and Lamp-2 (lysosome-associated membrane protein 2) respectively, compared with wild-type cells, indicating its augmented internalization in mutant cells. Moreover, when wild-type pancreatic islet cells were incubated with synthetic glycans, similar to those present on Glut-2 *N*-glycan branches, a profound reduction in cell-surface Glut-2 expression was observed after the addition of LacNAc and poly-LacNAc, whereas no reduction occurred using sucrose or LacNAc-bearing terminal sialic acid linkages. Thus, competitive inhibition of lectin–receptor binding for non-sialylated LacNAc structures from Glut-2 *N*-glycans was detected. The *N*-glycan structure of pancreatic Glut-2 from wild-type mice, as demonstrated from plant lectin-binding profiles, is a tetra-antennary complex type bearing little or no sialic acid and instead containing terminal galactose residues linked to LacNAc; the absence of Mgat4a results in a bi-antennary complex-type *N* glycan. Furthermore, when pancreatic islet sections from wild-type mice and *Mgat4a*-null littermates were analysed by fluorescence deconvolution microscopy, cell-surface co-localization of Glut-2 and Gal-9 was reduced among Mgat4a-deficient cells compared with control cells, reflecting diminished Glut-2 expression on the plasma membrane. Together, these findings imply that Mgat4a-mediated glycosylation of Glut-2 produces *N*-glycans specific for Gal-9 binding in pancreatic β-cells, which reduces the rate of Glut-2 endocytosis, thus facilitating Glut-2–Gal-9 interactions and sustaining glucose-stimulated insulin secretion. This mechanism provides an alternative pathway to counteract the pathological consequences of Type 2 diabetes [134].

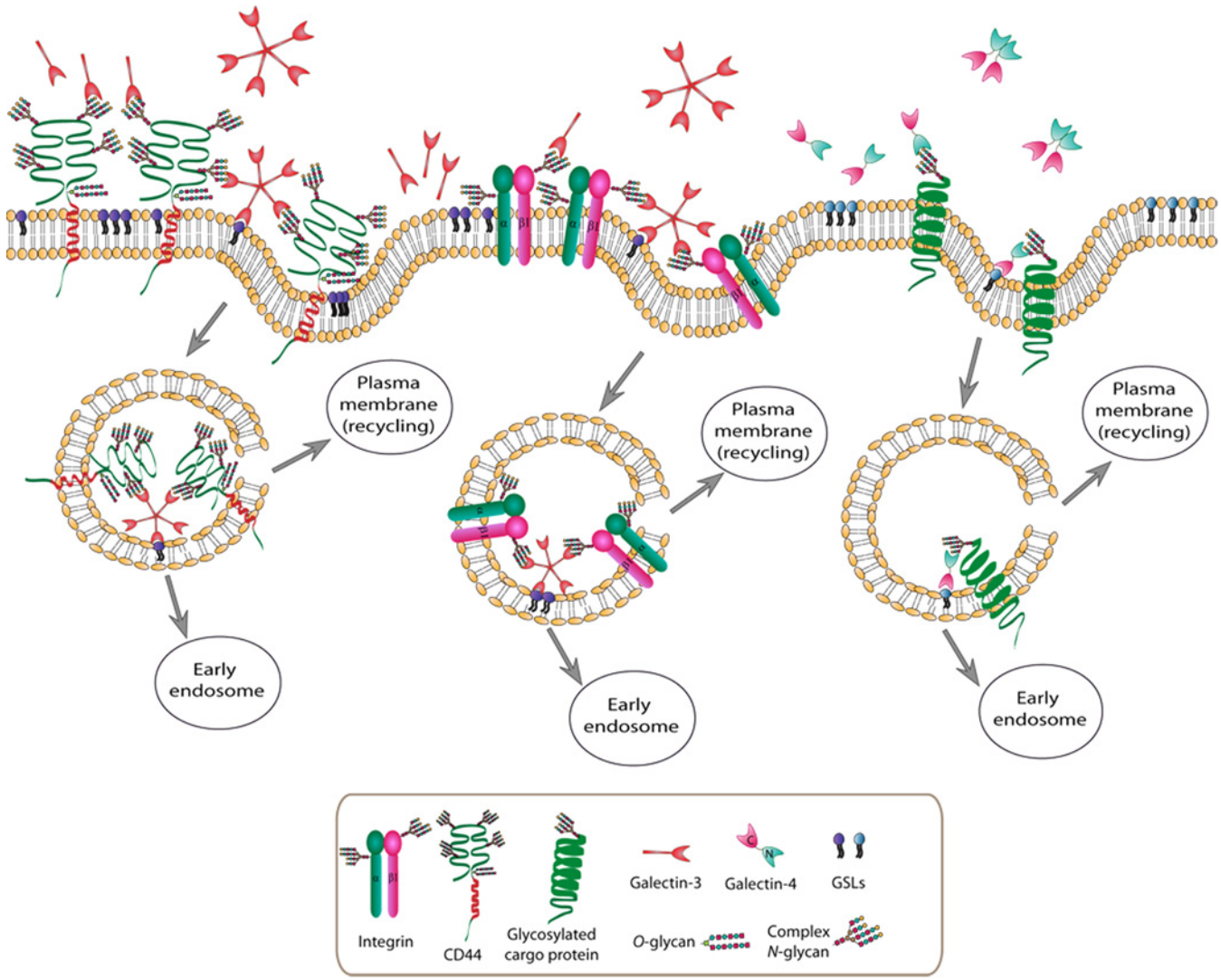

Figure 4 CLIC-mediated endocytosis induced by Gal-3 and Gal-4

Gal-3 and Gal-4 are synthesized in the cytoplasm and secreted to the extracellular milieu independently of the classical secretory pathway. At low concentrations, cell-surface Gal–glycan complexes are disrupted, and Gal-3 and Gal-4 drive the biogenesis of CLICs. Monomeric Gal-3 can bind to *N*-glycans on cell-surface glycoproteins such as CD44 and integrins. *N*-glycan-bound Gal-3 oligomerizes to form pentamers through its N-terminal domain. Gal-3 pentamers cross-link CD44 or integrins and GSLs to form tubular membrane invaginations in a clathrin-independent mechanism of endocytosis. Gal-4 is also internalized by this route with its, currently unknown, glycosylated ligands as cargo proteins.

Gal internalization coupled to different cargoes: a novel role in the biogenesis of clathrin-independent endocytosis

Co-clusters of Gal-3 and CD44 have been shown to internalize through a newly discovered mechanism [135]. This mechanism is likely to occur when diminished Gal concentrations at the cell surface allow Gal–glycan complexes to be disrupted and internalization of Gals proceeds. Gal-3 was first shown to trigger the GSL-dependent biogenesis of a morphologically distinct class of tubular and ring-shaped endocytic structures, termed CLICs (clathrin-independent carriers) (Figure 4). Thus, an integrated model for the function of Gal-3 and GSLs in cargo recruitment and in the biogenesis of CLICs was proposed: Gal-3 binds to the CLIC cargo protein CD44 and controls its GSL-dependent endocytosis, as well as that of β1 integrin [135]. In fact, CD44, cholera toxin and fluid-phase markers were the first cargoes shown to be internalized in CLICs [136], and these vesicles seem to represent the first station in a major physiologically important internalization route, arising directly from the plasma membrane, maturing into GPI (glycosylphosphatidylinositol)-enriched early endocytic compartments, and subsequently merging with early endosomes [137,138].

CLIC processes are particularly sensitive to GSL levels, because when GSLs are depleted, using inhibitors of glycosylceramide synthase, the occurrence of CLIC structures is significantly reduced. In mouse embryonic fibroblasts, both Gal-3 and Gal-4 conjugated to the fluid-phase marker HRP (horseradish peroxidase) decorated structures with CLIC morphology. Removal of the extracellular domains of glycosylated and non-glycosylated plasma membrane proteins with proteinase K or by treatment with inhibitors of protein *N*-glycosylation resulted in decreased Gal-3 binding, indicating that *N*-glycosylated proteins are required for Gal-3 recruitment to the plasma membrane [135]. CD44 and Gal-3 have been previously detected in CLIC-positive fractions by MS [139]. Furthermore, Gal-3 co-localized with endocytic structures that were positive for endogenous CD44,

and exogenous Gal-3 co-localized with endogenous CD44 at very early times of uptake, suggesting that Gal-3 and CD44 were internalized by the same endocytic carriers. CD44 uptake was strongly perturbed in cells treated with *N*-glycosylation inhibitors, and a CD44 mutant in which five *N*-glycosylation sites were eliminated failed to be internalized, demonstrating the importance of *N*-glycosylation in CD44 clustering and uptake. In addition, Gal-3 has been demonstrated to be required for CLIC formation as Gal-3 depletion using a specific siRNA reduced the formation of fluid-phase HRP-labelled CLICs. Moreover, although the amounts of cell-surface CD44 were not affected, CD44 uptake was strongly inhibited in Gal-3-depleted cells. In conclusion, this model predicts that monomeric Gal-3 is recruited to the cell surface through binding to glycosylated cargo proteins, such as CD44 and β1 integrin, then it oligomerizes and gains functional GSL-binding capacity, producing co-clustering of cargo proteins and GSLs, which generates mechanical stress to develop CLICs (Figure 4). It remains to be elucidated whether or not this mechanism also occurs for other Gals, and how local lectin concentrations at the cell surface dynamically modulate the ultimate fate of a membrane-associated Gal (lattice compared with internalization).

CONCLUSIONS

In summary, Gals have been shown to modulate a variety of cellular programmes through the formation of lectin–glycan complexes with glycosylated receptors that control receptor segregation, internalization and clustering. These Gal–glycan complexes selectively regulate the dynamics of glycosylated binding partners, both protein and lipid, limiting receptor internalization and maintaining downstream signalling sensitivity. Gal–carbohydrate interactions acquire various geometries depending mainly on the valency and presentation, and are critical membrane organizers, especially at sites of cell–cell contacts. Each Gal–glycan complex is likely to be a highly heterogeneous domain composed of multiple Gals plus glycoproteins and glycolipids (Figure 2A), which promotes not only homologous clustering but also heterologous interactions between different molecules such as clusters of differentiation (CD molecules), growth factor receptors, integrins or GSL. Interactions between CD45 and Gal-1 or Gal-3 on T-cells, mediated by both complex-type *N*-sugar chains and *O*-glycans on CD45, have been studied intensively, depicting a well-defined Gal–glycan complex where developmentally regulated glycosylation as well as expression of specific glycoforms of CD45 play a central role. Exogenous Gal-1 added to T-cells segregates CD43 and CD45 into different membrane microdomains and may control T-cell fate. Moreover, in T-cells, TCR and Gal-3 are involved in the control of molecular and cellular events that modulate the establishment of the immune synapse. On the other hand, Gal-1 and Gal-3 form complexes with VEGFR2, which influences cell-surface receptor retention and VEGF-dependent or independent signalling programmes on vascular cells. Lectin interactions at the cell surface are intricate because there are 15 mammalian Gals and redundancy may be one of the major mechanisms that operate in these signalling pathways, in addition to the contribution of other glycan-binding proteins such as C-type lectins and siglecs (sialic acid-binding, immunoglobulin-like lectins) [140]. Nevertheless, Gal-glycan complexes function both as a suppressor (e.g. TCR) or amplifier (e.g. VEGFR2 or EGFR) of receptor signalling serving as an environmental sensor to promote receptor function in a ligand-dependent or -independent fashion. Targeting *N*- and *O*-glycan biosynthesis or silencing expression of selected members of the Gal family may offer potential therapeutic applications, thus emphasizing the relevance of Gal–glycan regulation in cell signalling and homoeostasis.

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