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CELL SCIENCE AT A GLANCE

Galectins at a glance

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ABSTRACT

Galectins are carbohydrate-binding proteins that are involved in many physiological functions, such as inflammation, immune responses, cell migration, autophagy and signalling. They are also linked to diseases such as fibrosis, cancer and heart disease. How such a small family of only 15 members can have such widespread effects remains a conundrum. In this Cell Science at a Glance article, we summarise recent literature on the many cellular activities that have been ascribed to galectins. As shown on the accompanying poster, these include carbohydrate-independent interactions with cytosolic or nuclear targets and carbohydrate-dependent interactions with extracellular glycoconjugates. We discuss how these intra- and extracellular activities might be linked and point out the importance of unravelling molecular mechanisms of galectin function to gain a true understanding of their contributions to the physiology of the cell. We close with a short outlook on the organismal functions of galectins and a perspective on the major challenges in the field.

KEY WORDS: Endocytosis, Galectin, Glycosylation

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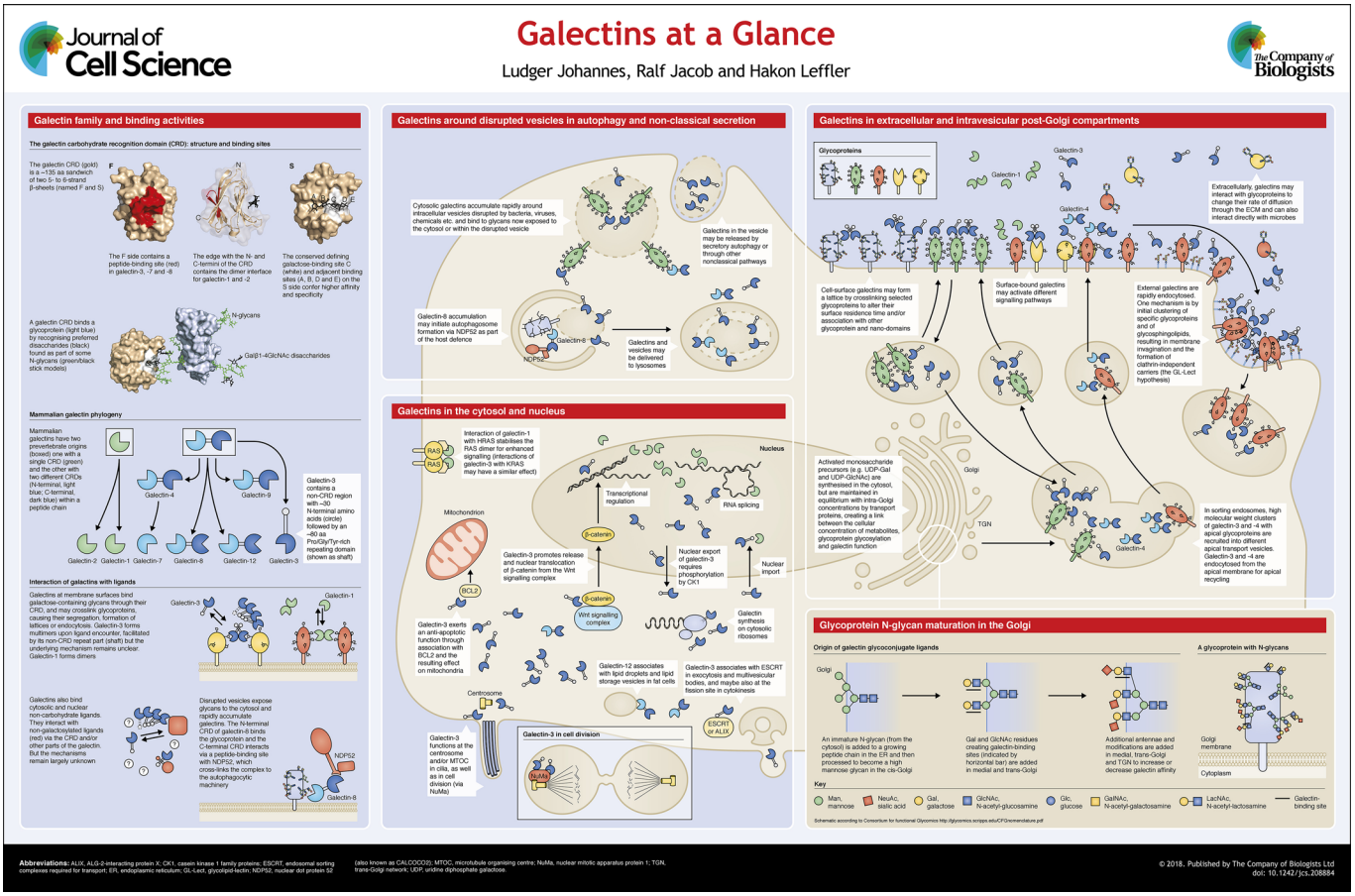
Introduction

Galectins were discovered based on their galactoside binding activity, in a quest to find proteins that decode complex cell-surface glycans. They were defined as a protein family based on conserved β-galactoside-binding sites found within their characteristic ∼130 amino acid (aa) carbohydrate recognition domains (CRDs) (Barondes et al., 1994). However, it was soon realised that galectins are synthesised as cytosolic proteins, reside in the cytosol or nucleus for much of their lifetime and reach their galactoside ligands only after non-classical secretion that bypasses



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the Golgi complex (Cummings et al., 2015). Indeed, galectins also interact with various non-galactose-containing binding partners, and their CRDs (or other parts) also have non-carbohydrate binding sites (Kim et al., 2013; Li et al., 2013) (see poster). While all cells express galectins, sometimes at cytosolic concentrations as high as 5 µM (Lindstedt et al., 1993), the expression pattern varies between cell types and tissues (Table 1).

Galectins are small soluble proteins that contain one or two CRDs but no other types of folded protein domains (Hirabayashi and Kasai, 1993). A core set of mammalian galectins are conserved from earlier vertebrate species, these comprise the 'prototype' galectin-1, -2 and -7, which contain one CRD, the chimeric galectin-3, and galectin-4, -8, -9 and -12, which are tandem repeat galectins with two CRDs (Houzelstein et al., 2004). Other galectins and galectin-related proteins are listed in Table 1.

Here, and in the accompanying poster, we exemplify the binding activities of galectins and their cytosolic and nuclear functions, as well as their capacity to: (1) recognise disrupted endosomal membranes; (2) be secreted via the non-classical pathway; and (3) modulate the activity of glycoproteins at the cell surface and inside the lumen of intracellular compartments. Because of space limitations, the focus of this article is on their cellular activities with the goal of stimulating interest in the interplay between intra- and extracellular galectin activities, and on the best studied galectins, mainly in human and mouse. We highlight how the understanding of molecular mechanisms will be critical to obtain confirmed models of galectin function. The organismal functions of galectins are only briefly summarised, including their tissue distribution (Table 1) and small molecule inhibitors (Box 1). A more detailed description of the carbohydrate structures to which galectins bind (Boxes S1 and S2) and of the main defects that are observed in galectin knockout mice (Table S1) are presented in the supplementary material.

Binding activities of galectins

The galectin CRD folds as a slightly bent β-sandwich (Di Lella et al., 2011; Leffler et al., 2004) (see poster). The concave S side forms a shallow groove that can approximately hold a tetrasaccharide (labelled A-B-C-D on the poster and Fig. S1). The defining galactose-binding site (C) that is conferred by a conserved sequence motif of about seven amino acids is localised within this groove. Weaker sites on either side of the galactose-binding site (including site E shown on the poster) enhance or decrease binding, thereby giving each galectin CRD its unique specificity and higher

Table 1. Tissue and species distribution of galectins and galectin-related proteins

Galectin	Tissue distribution in human and rodents	Species (closest relative[a])	Reference
Galectin-1	Expressed in many tissues and cell types	Vertebrates	Cooper, 2002
Galectin-2	Gastrointestinal tract, placenta	Birds and mammals, frogs, lizards (galectin-1)	Hutter et al., 2015; Oka et al., 1999
Galectin-3	Expressed in many tissues and cell types, especially prominent in macrophages and epithelial cells	Vertebrates	Cooper, 2002
Galectin-4	Intestine and stomach, uterine epithelial cells, blood vessel walls, hippocampal and cortical neurons	Vertebrates, except most birds	Froehlich et al., 2012; Huflejt and Leffler, 2004; Velasco et al., 2013
Galectin-5	Lung, kidney, reticulocytes, bone marrow, erythrocytes	Rat (galectin-9, C-CRD)	Cerra et al., 1985; Gitt et al., 1995; Lensch et al., 2006
Galectin-6	Gastrointestinal tract, liver, kidney, spleen, heart	Some mouse strains (galectin-4)	Gitt et al., 1998; Houzelstein et al., 2008; Houzelstein et al., 2013
Galectin-7	Gastrointestinal tract, stratified epithelia, skin, fetal heart	Mammals (galectin-4, N-CRD)	Magnaldo et al., 1998; Sato et al., 2002; Timmons et al., 1999
Galectin-8	Liver, kidney, cardiac muscle, lung, and brain	Vertebrates	Hadari et al., 1995
Galectin-9	T-lymphocytes, small intestine, liver, uterine epithelial cells, skin epidermis and oesophageal epithelium	Vertebrates	Froehlich et al., 2012; Lensch et al., 2006; Matsumoto et al., 1998; Wada et al., 1997
Galectin-10	Eosinophils	Primates, bats (galectin-4, N-CRD)	Abedin et al., 2003
Galectin-12	Adipose tissue	Vertebrates (galectin-8)	Hotta et al., 2001; Yang et al., 2004
Galectin-11[b]	Abomasal mucosa (ruminants), epithelial cells of bile ducts	Ruminants (galectin-4, N-CRD)	Dunphy et al., 2000; Young et al., 2012
Galectin-14[b]	Eosinophils	Ruminants (galectin-4, N-CRD)	Young et al., 2009
Galectin-15[b]	Uterus	Ruminants (galectin-4, N-CRD)	Gray et al., 2004
Galectin-11 and others[b]	Placenta	Primates (galectin-4, N-CRD)	Than et al., 2004; Than et al., 2009
GRP[c]	B-lymphocytes, bursa of Fabricius	Vertebrates (galectin-8 or -12, C-CRD)	Houzelstein et al., 2004; Manning et al., 2018
GRIFIN[d]	Eye lens	Vertebrates (galectin-2)	Caballero et al., 2018; Manning et al., 2018

[a]The main group within mammals is shown for galectins with limited species distribution. The closest related CRD among those conserved in most vertebrates is given.

[b]In the human placenta, a cluster of genes on chromosome 19 encode single-CRD galectins, all related to galectin-7, -10 and the N-terminal CRD of galectin-4 (also on chromosome 19); these include galectin-11, -13, -15 and potentially others. In ruminants, galectins with closely related sequences have been given overlapping names, despite their different tissue expression. Thus, it cannot easily be determined which are orthologues and which are paralogues.

[c]This galectin-related protein, termed HSPC159, was discovered as a clone from haematopoietic stem/progenitor cells (HSPCs). GRP does not have galactose binding activity and has a highly conserved sequence throughout vertebrates; it is best characterised in chicken.

[d]This protein was discovered in rat eye lens. It is found throughout vertebrates, with a highly variable sequence. The 7 amino acid galectin motif is disrupted in mammals, but almost complete in birds, and complete in fish and amphibians, suggesting it is a functional galectin in some species, as shown for chicken and zebrafish.

C-CRD, C-terminal carbohydrate recognition domain; N-CRD, N-terminal carbohydrate recognition domain.

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Box 1. Galectin inhibitors

The roles of galectins in cellular and pathophysiological processes has inspired the search for galectin inhibitors, both as potential drug leads, and as experimental tools in cell biology. A wide array of small-molecule inhibitors has been reviewed recently (Campo et al., 2016). Among these, the most potent inhibitors are based on disaccharide derivatives that fit into the core site (C-D), and with aromatic extensions that project into sites B and E, thereby reaching affinities in the low nM range. The synthetic disaccharide thiodigalactoside (Galβ1-S-1βGal) projects a glycotope for site C-D (see Box S1 and poster), which is very similar to that used by natural disaccharides (Leffler and Barondes, 1986). Thus, derivatising both Gal residues with aromatic extensions allows favourable interactions (K_d around 2 nM) with galectin-3. The compound TD139 has shown *in vivo* efficacy in animal models of fibrosis and angiogenesis (Chen et al., 2017; Delaine et al., 2016) and is currently in clinical trials against idiopathic lung fibrosis, while another compound showed efficacy against insulin resistance in obese mice (Li et al., 2016). A large number of compounds are currently being developed to optimise the selectivity among galectins and their pharmacokinetic properties (Rajput et al., 2016). The compounds that are based on disaccharides are relatively water soluble and fail to be taken up well across membranes, making them not available orally in animals. Recently, a new class of galectin inhibitors that contain only one sugar residue was developed (Zetterberg et al., 2017), which, for the first time, constitute membrane-permeable and orally available potent (K_d in the low nM range) galectin inhibitors. Plant-derived polysaccharides, such as modified citrus pectin (MCP) or galactomannans, have also been proposed to act as pharmacological inhibitors of galectin-3 (Demotte et al., 2014; Glinsky and Raz, 2009; Inohara and Raz, 1994; Traber and Zomer, 2013) and some have reached clinical trial (Girard and Magnani, 2018). However, they appear to interact poorly or not at all with the canonical galectin carbohydrate-binding site (Stegmayr et al., 2016). In the pectin-derived saccharides, galactose residues occur mainly in Galβ1-4Gal configuration (Zhang et al., 2017), which fits only poorly in the canonical galectin disaccharide-binding site (C-D). In galactomannans, the main galactoses are found in 6-linkage to mannose (Miller et al., 2012), with only weak binding to galectins (K_d in the mM range) (Stegmayr et al., 2016). Interaction at other sites has been proposed (Miller et al., 2016, 2009; Zhang et al., 2017). Another issue is their possible effects on cellular components in addition to galectins (Fan et al., 2018).

affinity for larger glycoconjugates (Hirabayashi et al., 2002; Kamili et al., 2016; Leffler et al., 2004; Salomonsson et al., 2010a). Thus, while galectin CRDs typically bind galactose by themselves with affinities in the millimolar range, they bind the common disaccharide N-acetyl-lactosamine with a mid-micromolar affinity and may bind to their preferred glycoprotein with sub-micromolar affinity (see Boxes S1 and S2 for a more detailed discussion).

Of note, binding sites on the CRD for non-carbohydrate-containing ligands, such as those found in the cytosol and nucleus (described below), have also been identified. The best characterised example is the C-terminal CRD of galectin-8, where a binding site on the F-side (see poster) confers a specific affinity for the protein NDP52 (also known as CALCOCO2) (Kim et al., 2013; Li et al., 2013). A similar site in galectin-3 binds to its own N-terminal domain (Ippel et al., 2016). Sites at the edge of the CRDs confer the dimerisation of galectin-1, -2 and -7.

Even though galectins are largely monomeric in solution at physiological concentrations (Lepur et al., 2012; Salomonsson et al., 2010b), most are able to crosslink glycoconjugate ligands and cause their aggregation. For example, galectin-1 forms a weak dimer (K_d in low micromolar range) (Di Lella et al., 2011; Salomonsson et al., 2010b) and dimerisation may be enhanced upon ligand encounter. Galectin-3 is monomeric up to high concentrations (100 μM), but can aggregate with ligands and agglutinate cells at much lower concentrations, which is promoted by its N-terminal non-CRD region (Hsu et al., 1992; Massa et al., 1993) via an oligomerisation mechanism that is not yet completely understood (Ahmad et al., 2004; Barboni et al., 1999; Birdsall et al., 2001; Kuklinski and Probstmeier, 1998; Lepur et al., 2012; Nieminen et al., 2007; Yang et al., 1998) (see poster and Box S3). Galectins with two different CRDs may form non-covalent dimers to achieve an increase in valency (Kamili et al., 2016).

Multivalency is required for many of the biological effects of galectins, especially when they encounter the outside of cells, as it permits not only the crosslinking of ligands to induce signalling pathways and the formation of cell surface lattices, but also ligand endocytosis (see poster). Affinity enhancement by multivalency can be very strong for other lectin families and is termed the cluster glycoside effect (Lee and Lee, 2000; Lundquist and Toone, 2002). However, this effect appears to be modest or absent for galectins (Leppänen et al., 2005; Salomonsson et al., 2010b; Stowell et al., 2009).

It has been suggested that galectins may participate in liquid-liquid phase-separated structures (droplets) at the cell surface or in intracellular locations, based on weak interactions such as those found with terminal saccharide motifs (Banani et al., 2017; Dennis, 2015). Another interesting proposed role of weak binding is for rapid sampling of receptors (Kasai, 2006).

Galectins in the cytosol and nucleus

Galectins interact with a panoply of cytosolic and nuclear ligands that do not appear to be functionally linked (Haudek et al., 2010) (see poster). Galectin-1 and galectin-3 interact with the small GTPases HRAS (Paz et al., 2001) and KRAS (Shalom-Feuerstein et al., 2008), respectively. In one proposed mechanism, galectin-1 binds to and stabilises RAS dimers to positively regulate GTP-HRAS nanoscale signalling hubs (Blaževitš et al., 2016).

Galectin-3 associates with basal bodies and centrosomes, and its absence results in disorganisation of microtubules and perturbation of epithelial morphogenesis of primary cilia (Koch et al., 2010) or motile respiratory epithelial cilia (Clare et al., 2014). Moreover, galectin-3 associates with nuclear mitotic apparatus protein (NuMa), a key mitotic regulator required for the establishment and cohesion of the spindle pole (Magescas et al., 2017). Expression of mutant NuMA blocked in O-GlcNAc glycosylation results in aberrant spindle pole formation. This mutant does not co-immunoprecipitate galectin-3, which suggests that the galectin-3–NuMA interaction depends on O-GlcNAc glycosylation (Magescas et al., 2017). The exact mechanism remains unclear as O-GlcNAc itself does not bind galectin-3.

Galectin-3 also interacts with the endosomal sorting complex required for transport (ESCRT). Yeast two-hybrid analysis revealed interaction of the N-terminal domain of galectin-3 with the ESCRT component ALG-2-interacting protein X (Alix) (Chen et al., 2009), which promotes T-cell receptor downregulation, affects intracellular trafficking of the epidermal growth factor receptor (Liu et al., 2012) and release of human immunodeficiency virus (HIV)-1 (Wang et al., 2014). ESCRT components have fundamental roles in the biogenesis of multivesicular bodies (MVBs), which may provide opportunities to recruit galectin-3 into exosomes that are released from MVBs. Their presence in exosomes has been shown for galectin-3 (Fei et al., 2015; Thery et al., 2001; Welton et al., 2010) and galectin-9 (Keryer-Bibens et al., 2006).

Other examples of cytosolic functions for galectins include the association of galectin-3 with β-catenin and the Wnt signalling

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pathway (Shimura et al., 2005) and with Bcl2 for the regulation of apoptosis (Harazono et al., 2014b). Here, the NWGR motif in galectin-3 has been recognised to be anti-apoptotic and implicated in its association with both Bcl2 and Ras (Harazono et al., 2014a). However, it is important to note that this sequence is also part of the canonical galactose-binding site (Salomonsson et al., 2010a).

Galectin-3 shuttles between the cytoplasm and the nucleus. Nuclear localisation sequences were identified in murine galectin-3 [(253)ITLT(256); Davidson et al., 2006] and human galectin-3 [(223)HRVKKL(228); Nakahara et al., 2006], and the latter was shown to bind to importin-α for passage through nuclear pores. In addition, a leucine-rich nuclear export signal (NES) is found at positions 241-249 of murine galectin-3 (Tsay et al., 1999). All these sequences are within the CRD. Yet, the N-terminus of galectin-3 may also contribute to active nuclear transport. Indeed, phosphorylation of galectin-3 at Ser6 by casein kinase 1 (Huflejt et al., 1993) plays a role in its nuclear export and its anti-apoptotic activity (Haudek et al., 2010).

In the nucleus, galectin-1 and galectin-3 are involved in nuclear splicing of pre-mRNAs and in gene expression (Dagher et al., 1995; Vyakarnam et al., 1997). Both galectins have been shown to be functionally redundant splicing factors for mRNA processing and nuclear export (Wang et al., 2006). Galectin-3 assembles with small nuclear ribonucleoprotein (snRNP) particles in nuclear extracts. Here, it was identified in the earliest detectable mammalian prespliceosome complex (E-complex) (Haudek et al., 2016), and the heterogeneous nuclear ribonucleoprotein complex (H-complex) (Fritsch et al., 2016). These complexes initiate spliceosome assembly and the splicing cascade (Michaud and Reed, 1991). In the cytosol of pancreatic cancer cells, galectin-3 also stabilises *MUC4* mRNA by interacting with the heterogeneous nuclear ribonucleoprotein L (Coppin et al., 2017). Galectin-3 has furthermore been implicated in the regulation of transcription through various mechanisms (see Kang et al., 2016 and references therein).

Galectins at disrupted vesicles with roles in autophagy and non-classical secretion

Galectins rapidly accumulate around disrupted endocytic vesicles or compartments. This appears to be due to the binding of cytosolic galectin to glycans, which are normally present in the lumen and, upon disruption, are exposed to the cytosol. This phenomenon was first discovered for galectin-3 on *Shigella*-containing vacuoles that undergo lysis (Paz et al., 2010) (see poster). Subsequently, it was also observed for galectin-8 on Salmonella-containing vacuoles (Thurston et al., 2012), galectin-3 on adenovirus-containing endosomes (Maier et al., 2012), and for galectin-1 and galectin-9 (Thurston et al., 2012). Galectins also accumulate around endosomes that have been disrupted in other ways, for instance by protein aggregates in neurodegenerative disease (Flavin et al., 2017; Jiang et al., 2017), by chemicals (Aits et al., 2015) or by nucleic acid transfection reagents (Wittrup et al., 2015). One function of this phenomenon is to link vesicle disruption to selective autophagy; here, the N-terminal CRD of galectin-8 interacts with glycoconjugates, whereas the C-terminal CRD interacts with the pathogen-specific autophagy receptor NDP52 (Boyle and Randow, 2013; Kwon and Song, 2018). Galectin-3 accumulation has been proposed to inhibit the galectin-8-mediated link to autophagy (Cheng et al., 2017). Galectins also interact with the tripartite motif-containing (TRIM) proteins, which are another class of receptors for selective autophagy (Chauhan et al., 2016).

The accumulation of galectins around disrupted vesicles is very rapid (within minutes) and easy to detect with immunohistochemistry or when using GFP-conjugated galectins (Paz et al., 2010). Hence, galectins have become a useful tool for detecting vesicle disruption in cell culture and *in vivo* (Aits et al., 2015), which can be exploited to assess the intracellular activity of galectin inhibitors (Stegmayr et al., 2016) (see also Box 1).

The non-classical secretion of galectins was shown to require their galactoside-binding activity (Seelenmeyer et al., 2005); this has led to the suggestion that accumulation around disrupted vesicles and binding to luminal glycans might provide a mechanistic link between autophagy and galectin secretion (Hughes, 1999). However, in another study, maturation of N-linked glycoprotein was not required for galectin-3 transport from the cytosol to the extracellular space, and secreted galectin-3 was predominantly free and not packaged into extracellular vesicles (Stewart et al., 2017), indicating that further studies are required to settle this matter. For galectin-4 secretion, a link to Src-mediated tyrosine-phosphorylation has been proposed (Ideo et al., 2013). Thus, the mechanism for the non-classical secretion of galectins remains unclear.

Galectins in extracellular and intravesicular compartments

Upon their non-classical secretion, galectins enter the extracellular space, where they encounter ligands such as galactose-containing glycoproteins and glycolipids (see poster). Galectins can re-enter cells by endocytosis (Furtak et al., 2001) and populate endocytic and recycling compartments (Stechly et al., 2009; Straube et al., 2013).

According to the glycolipid-lectin (GL-Lect) hypothesis, binding of galectin to glycosylated cargo proteins, such as CD44 and α5β1 integrin, triggers galectin oligomerisation, which then enables their interaction with glycosphingolipids (Box S2) and the generation of membrane curvature, leading to the formation of tubular endocytic pits from which clathrin-independent endocytic carriers are formed (Johannes et al., 2016; Lakshminarayan et al., 2014) (see poster).

Galectins have also been described to counteract endocytic uptake by forming lattices at the plasma membrane (Brewer et al., 2002; Demetriou et al., 2001; Dennis, 2015; Dennis and Brewer, 2013; Dennis et al., 2009; Di Lella et al., 2011; Gupta et al., 1996; Kamili et al., 2016; Sacchettini et al., 2001; reviewed in Nabi et al., 2015). The balance between stimulation of endocytosis or the formation of an inhibitory lattice may depend on the specificity, structure and concentration of both the galectins themselves, and their glycoprotein and glycolipid ligands. A highly interesting link to the metabolic state of cells has been proposed, which is mediated by the intracellular levels of GlcNAc and UDP-GlcNAc that affect quantity and branching of complex N-glycans and so regulate galectin binding and the galectin lattice (Lau et al., 2007).

The T168N mutation in the interferon-γ receptor (IFN-γR), which causes Mendelian susceptibility to mycobacterial diseases syndrome (MSMD) (Kong et al., 2010), illustrates how abnormal interactions with galectins affect receptor function. This mutation creates a new N-glycosylation site, in addition to the six that are normally present; this draws the receptor from lipid nanodomains into actin nanodomains, thereby reducing its signalling function (Blouin et al., 2016). This also demonstrates that a dysregulation of the galectin system may predispose to disease. A similar interplay between actin and galectins has also been described for the T-cell receptor (Chen et al., 2007).

Galectins regulate the trafficking of intravesicular glycoprotein cargoes. Galectin-3 and galectin-4 accumulate in endosomal organelles following their internalisation from the cell surface

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(Stechly et al., 2009; Straube et al., 2013). Alternatively, a direct route from the cytosol into the lumen of endosomes could be possible, but this has not yet been shown. These endosomes serve as platforms for the sorting of cargo molecules into transport vesicles destined for the apical membrane of epithelial cells. Galectin-4 specifically interacts with lipid raft-associated glycoproteins, whereas galectin-3 binds to the non-raft fraction (Delacour et al., 2006, 2005). Depletion of the two galectins results in basolateral mis-sorting of the corresponding glycoproteins, suggesting an involvement of galectins in apical protein sorting (Delacour et al., 2006, 2005). Galectin-3-mediated apical sorting comprises pH-dependent cargo binding (von Mach et al., 2014) and cargo crosslinking into high-molecular weight clusters in a post-Golgi compartment (Delacour et al., 2007). Galectin-4-dependent sorting depends on the exposure of complex N-glycans on apical glycoproteins (Morelle et al., 2009). Both galectins escort their ligands to the apical cell surface, where they enter the apical milieu and are then reinternalised into endosomal organelles for a new round of apical protein sorting (Stechly et al., 2009; Straube et al., 2013) (see poster). Such a pathway is further supported by evidence for retrograde trafficking of galectin-9 from the plasma membrane to the Golgi (Mishra et al., 2010), which suggests participation of this galectin (and possibly others) in sorting events at the trans-Golgi network (TGN).

Extracellular galectins also induce signals from the cell surface and modify interactions with and within the extracellular matrix, as well as with microbes (Arthur et al., 2015; Di Lella et al., 2011; Gordon-Alonso et al., 2017; Thiemann and Baum, 2016). Most studies described here have considered only the common galactoside binding ability of galectins, but not the finer details in their specificity (described in Box S1). The role of N-glycan branching and extension with poly-N-acetyl lactosamines in lattice formation has been analysed and modelled in detail (Kamili et al., 2016; Nabi et al., 2015). The role of higher N-glycan branching for galectin-3 binding and intracellular routing of transferrin has also been demonstrated (Carlsson et al., 2013). Sialylation at the 3-position of galactose, which gives high affinity binding to galectin-8, directs the intracellular trafficking of this galectin (Carlsson et al., 2007). The inhibition of galectin-1 binding by sialylation at the 6-position of galactose has been suggested to make Th1 cells resistant to apoptosis (Di Lella et al., 2011).

Links between the extracellular and cytosolic functions of galectins

The cytosolic and nuclear localisation and functions of galectins (Haudek et al., 2010) have been known almost as long as the extracellular ones (Di Lella et al., 2011), and this raises the question of whether the intra- and extracellular functions of galectins are connected by a regulatory system. Early evidence for this came from the observation that galectin-1 switches from a predominantly intracellular localisation in cultured myoblasts to a predominantly extracellular localisation upon their differentiation into myotubes (Cooper and Barondes, 1990). In developing mammary gland epithelial cells, galectin-1 was found mainly in the cytosol and nucleus of cells of the end bud, whereas it localised to the cell surface or extracellularly in duct cells (Bhat et al., 2016). Furthermore, galectin-1 binding to glycoconjugates was increased in the duct cells, with evidence that this interaction could ‘draw’ the galectin to an extracellular location (Bhat et al., 2016). Such a mechanism could provide a regulatory loop, whereby extracellular galectin-1-binding glycoconjugates reduce galectin concentration and associated functions in the cytosol and/or nucleus.

Considerations at the organismal level

A highly interesting and plausible picture of the physiological and pathophysiological galectin functions in inflammation, immunity, cancer and many other diseases (Sciacchitano et al., 2018) has emerged from biochemical and cellular studies as described above, as well as from evidence from whole organisms (Table 1), in which galectins or their ligands were manipulated using genetic techniques, galectin inhibitors or galectins themselves (Table S1). As a detailed discussion is beyond the scope of this review, we pose here three fundamental questions.

What is the role of galectins in serum?

The concentration of galectins in serum or plasma is typically in the high picomolar range, whereas most cellular studies have been performed at much higher concentrations. Thus, it is not likely that galectins act at a distance, similar to circulating hormones. Instead, their presence in serum may result from leakage from tissue, as has been found for many other proteins (Landegren et al., 2018) and serum levels of galectins could therefore be useful as diagnostic markers for disease. A correlation between galectin-3 levels and heart disease has indeed recently emerged (Gehlken et al., 2018). Furthermore, galectin-driven homotypic aggregation and/or adhesion of cancer cells to endothelial cells has been proposed to lead to increased metastasis (Glinsky et al., 2001; Sindrewicz et al., 2016). It has also been shown that at concentrations as low as 0.3 nM, galectin-3 stimulates sugar-dependent CD44 endocytosis (Lakshminarayan et al., 2014), whereas galectin-7 affects the endocytosis of E-cadherin in a sugar-independent manner (Advedissian et al., 2017). These findings suggest that some galectin-mediated cellular activities might indeed be sufficiently sensitive to respond to serum levels of galectins.

Can galectins from one cell act on other cells?

Adoptive transfer of bone marrow between galectin knockout and wild-type mice clearly showed that galectin-1 from cancer cells has angiogenic and immunosuppressive effects on the surrounding cells (Méndez-Huergo et al., 2017). Galectin-3 from myeloid cells also affects the growth of cancer cells and induces insulin resistance in fat cells (Li et al., 2016). Hence, galectins can have both autocrine and paracrine functions.

Do galectins have intra- and extracellular functions at the organismal level?

Most of the descriptions of galectin function in whole organisms consider the carbohydrate-binding activity of galectins, and have been suggested to take place either within the extracellular space, at the cell surface and/or in intracellular trafficking pathways (Blidner et al., 2015; Thiemann and Baum, 2016). However, as discussed above, galectins are rapidly endocytosed and directly traffic along both endocytic and exocytic pathways (Furtak et al., 2001; Lakshminarayan et al., 2014; Schneider et al., 2010; Straube et al., 2013). Thus, the cellular expression and release of galectins can modify these trafficking properties, possibly by setting an optimum level of galectin in the combined intra- and extracellular compartments of an organism. This may result in direct galectin-induced signalling (e.g. induction of apoptosis in T-cells) or galectin-regulated cell surface exposure and lateral organisation of glycosylated receptors, such as T-cell, VEGF, TGFβ and insulin receptors, along with their subsequent effects, including induction of angiogenesis, lymphangiogenesis, fibrosis in chronic inflammation and other mainly immune-regulatory functions (Blidner et al., 2015; Thiemann and Baum, 2016) (Table S1).

By contrast, the contribution of the cytosolic and nuclear activities of galectins to their organismal functions have been considered to a much lesser extent. The accumulation of galectins around damaged vesicles has been shown to occur also on cells within the organismal context (Aits et al., 2015), and thus may be a useful marker for vesicle-disrupting activities *in vivo*, as observed in neurodegenerative disease (Flavin et al., 2017; Jiang et al., 2017). Another elegant example is the role of galectin-3 in organising airway cilia and other cilia within the living organism (Clare et al., 2014), demonstrating an organismal role for the association of galectin-3 with centrosomes. These roles of galectins may also be connected to their extracellular activities, as described above, and have stimulated the development of galectin inhibitors as therapeutics (Box 1). Such drugs have been used to inhibit the immunosuppressive and angiogenesis-inducing effects of galectin-1, galectin-3 and galectin-9 in cancer (Blidner et al., 2015), to prevent unwanted angiogenesis in the eye (Chen et al., 2017) and to reduce fibrosis in chronic inflammation (Delaine et al., 2016). Anti-inflammatory galectin inhibitors have already reached the clinical development stage (https://galecto.com/).

Conclusions and perspectives

Although the galectin field has come a long way in the past decade, a number of aspects remain incompletely understood, such as the molecular mechanisms of their actions in intra- and extracellular space and the relationship between these activities, as well as any associated regulatory loops. Furthermore, the structural aspects of galectin function, notably those linked to their oligomerisation, are also not fully known and require further work. Another exciting challenge in the field of cellular glycobiology is to elucidate the quantitative aspects of galectin distribution in cells and tissues, and the redundancies and specificities of galectins in health and disease. Addressing these issues will help to demystify the many cellular and organismal functions of galectins and pave the way to future therapeutic strategies for associated diseases and conditions.

Competing interests

H.L. is co-founder and shareholder in Galecto Biotech AB.

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Cell science at a glance

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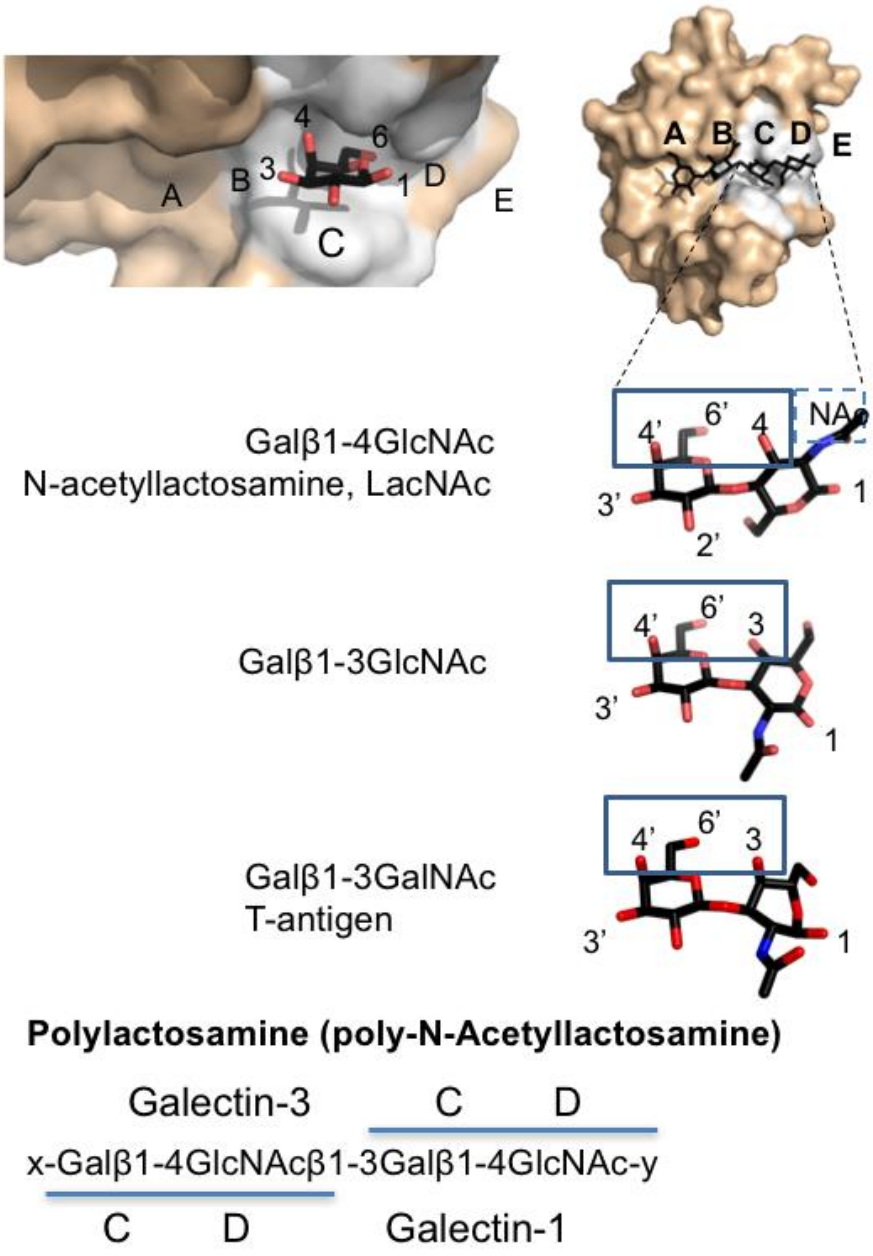

Fig. S1. Galectin carbohydrate-binding site. **Top:** Close up of the conserved (white) defining galactose-binding site, and whole CRD with a bound tetrasaccharide (black stick model). Subsites are indicated as A-E as described in text. **Middle:** Chemical designation, and stick models of three common disaccharides in mammalian glycoconjugates. Hydroxyl groups on the disaccharide are numbered according chemical convention, with a prime symbol (') for those on the non-reducing end galactose. The galectin-binding glycotope is boxed. **Bottom:** Polylactosamine with main binding sites for galectin-1 and -3.

Box S1. Carbohydrate-binding specificity of mammalian galectins

In mammalian cells, galectins bind large glycocojugates, such as glycoproteins and glycolipids (poster). A fairly simple well defined small epitope (glycotope) is a necessary determinant for canonical galectin-binding (Kamili et al., 2016; Leffler and Barondes, 1986; Leffler et al., 2004), but it is not sufficient because interaction with other parts of the glycoconjugates are needed to reach a biologically significant affinity of in the upper nM or lower µM range.

Most of such a galectin glycotope (boxed part of three sample disaccharides in Fig. S1) is contributed by a galactose residue that fits into the galectin-defining binding site and reaches mM affinity by itself (site C as shown in poster and Fig. S1). One more hydroxyl group in a precise location (in site D), which is contributed by an adjacent sugar residue, is required to boost affinity to the 100 µM range.

In a natural glycoconjugate, the disaccharide in sites C-D is linked to other saccharides, peptides, lipids, or other moieties through several hydroxyl groups (numbered in Fig. S1). If such modifications are found within the galectin defining glycotope (boxed in Fig. S1), they will block binding for all galectins. Thus, the common modification with sialic acid (e.g. NeuAc) at hydroxyl 6 of the Gal residue (labeled 6' by chemical convention; see legend to Fig. S1) in site C will prevent binding (Amano et al., 2003; Kamili et al., 2016; Leffler and Barondes, 1986; Thiemann and Baum, 2016). In other cases, a Fuc residue on positions 3 or 4 on the adjacent GlcNAc in site D will block binding. Thus, the Le^x and Le^a modifications, required by selectins, will prevent galectin-binding.

The 3'-position of the galactose residue is outside the galectin-binding epitope. Different galectins have different preference for additional carbohydrates here, often found as terminal modifications of complex glycans (Hirabayashi et al., 2002; Kamili et al., 2016; Leffler and Barondes, 1986; Leffler et al., 2004). For example, addition of sialic acid at this position is tolerated by galectin-1 and galectin-3, but prevents the binding of galectin-2, whereas it strongly enhances binding of the galectin-8 N-terminal CRD (Ideo et al., 2011). Modification by GalNAcα or Galα at this position, as found in blood group A, Forssman, and Galili antigens may boost affinity 10- to 25-fold for some galectins, but not for others. Such modifications occur at high abundance in specific locations, e.g. erythrocytes and intestine in humans; (Finne et al., 1989; Viitala et al., 1982), but are less common than sialylation that is typically found on all cells in culture. Additions at position 2 may also affect binding, most commonly Fucα that appears to be preferred by galectin-7 (www.functionalglycomics.org).

A GlcNAcβ at 3'Gal may be further modified with another Gal to form a polylactosamine structure (a repeat of LacNAc disaccharides) (Fig. S1, bottom). These are often mentioned as preferred galectin ligands, because they exhibit enhanced binding in some assays (Leppanen et al., 2005; Merkle and Cummings, 1988), and fit with other evidence regarding the galectin lattice (Brewer et al., 2002; Nabi et al., 2015). However, two different mechanisms may exist. If the GlcNAcβ at 3' Gal is tolerated, as with galectin-3, it means that an internal LacNAc residue can be bound in site C-D, and another LacNAc linked to 3' Gal may interact in site A-B, where it may enhance binding by about 10-fold. If the GlcNAcβ at 3' Gal is not well tolerated, as is the case for galectin-1, only terminal LacNAc residues may bind in site C-D instead, and site A-B remains unoccupied. This may make the binding site extend further out from a protein or surface, which may result in an apparent affinity enhancement in assays where short glycans give rise to poor binding (Leppanen et al., 2005).

In natural glycoconjugates, position 1 of the disaccharide in site C-D is linked to additional saccharides, lipids and/or proteins (Fig. S1). These moieties may interact at the edge of the galectin carbohydrate-binding groove or the back of the galectin (poster), loosely defined as site E (Salomonsson et al., 2010), and strongly enhance affinity, or in other cases potentially prevent binding.

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Box S2. Natural glycoconjugate ligands of galectins

Here, we will describe how the binding specificities described in Box S1 play out for the main classes of mammalian glycoconjugates.

N-glycans. Galβ1-4GlcNAc (LacNAc) is the most common potential galectin-binding site within glycoproteins, and occurs mainly in N-glycans (but also some O-glycans); mutant CHO cells lacking Gal in N-glycans are not bound by most galectins (Patnaik et al., 2006). By itself LacNAc has a K_d of around 100 μM for galectin-binding, whereas glycoproteins bind at 1 μM or lower. How is the additional affinity achieved? A likely source are the moieties to which the disaccharide is linked. They consist of the rest of the N-glycans (or O-glycans) and the protein to which they are attached. Thus, either some other feature of more complex branched N-glycans, or the protein itself must contribute, but much remains to be done to define these interactions better. The presence of three or more antennae clearly leads to increased affinity for galectins, as determined both for soluble serum glycoproteins (Carlsson et al., 2013) and in cells (Nabi et al., 2015). One commonly given explanation is that one particular antenna is often elongated with its polylactosamine to give rise to the higher affinity (Nabi et al., 2015). However, serum glycoproteins do not have polylactosamines, although multiple antennae are still required for galectin-binding. Galβ1-3GlcNAc (see the middle disaccharide in Fig. S1) by itself has an equal or lower affinity for most galectins compared to LacNAc. However, its position 1 that links it to further moieties has a different direction compared to that in LacNAc (compare the two saccharides in Fig. S1), and it thus may provide an indirect source of specificity (Leffler et al., 2004). The precise site on a glycoprotein at which the N-glycan is attached may also be important, but this aspect remains largely unexplored.

O-glycans. Galβ1-3GalNAc (bottom disaccharide in Fig. S1) occurs in O-glycans and in glycosphingolipids. It is identical to Galβ1-3GlcNAc, except that the 4-OH has a different direction (axial instead of equatorial). Galβ1-3GalNAc (and its 3'-sialylated form) have attracted much attention, as it constitutes the T-antigen, which is often related to cancer (Sindrewicz et al., 2016). By itself, Galβ1-3GalNAc has poor affinity for galectin-1 and galectin-3 (Bian et al., 2011), but exhibits a higher affinity for some other galectins including the N-terminal CRD of galectin-8 (Carlsson et al., 2007; Ideo et al., 2011). The latter has a low μM affinity for the 3-sialylated T-antigen, and it may be one of its preferred ligands (Carlsson et al., 2012). Galectin-1 and galectin-3 have also been proposed to more strongly bind T-antigen carrying mucin-like polypeptides, but apparently this requires the involvement of additional interactions beyond the disaccharide (Bian et al., 2011).

Glycolipids. Most glycosphingolipids contain a lactose (Galβ1-4Glc) disaccharide closest to the lipid part (ceramide). However, in many glycolipids, this is extended on 4' Gal and, hence, blocked for galectin-binding. The binding of galectins to GM1 ganglioside has been proposed based on indirect evidence from effects on cholera toxin binding to cells (Boscher et al., 2012; Kopitz et al., 1998), but is poorly supported by direct evidence. In the GM1 pentasaccharide (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-), site C-D can only bind the terminal Galβ1-3GalNAc because the internal Gal is blocked by the linkage to position 4, and, hence, the rest of the GM1-molecule (NeuAcα2-3Galβ1-4Glc-Cer) must be in site E. How well GM1 binds to galectins remains unclear, however, as its pentasaccharide did not bind to galectin-1, and only weakly to galectin-3 as determined by isothermal titration calorimetry (Bian et al., 2011). Furthermore, in glycan-arrays, the GM1 ganglioside pentasaccharide usually does not show any binding activity for galectins (data available on www.functionalglycomics.org/).

Box S3. Galectin-3 structure and self-association

Even at high concentrations, galectin-3 is a monomer, but may quickly aggregate in the presence of ligands, giving it the ability to agglutinate cells, induce signals, as well as to bind cooperatively. The mechanism of this self-association of galectin-3 is not completely clear despite many studies addressing the issue (Barboni et al., 1999; Birdsall et al., 2001; Halimi et al., 2014; Kuklinski and Probstmeier, 1998; Lepur et al., 2012; Lin et al., 2017; Nieminen et al., 2007; Yang et al., 1998). In addition to the CRD, galectin-3 has two non-CRD parts (i.e. 30 aa N-terminal domain, and about 12 repeats of 6-12 aa), both of which contribute to its oligomerization. Interactions between CRDs appear to involve and block the carbohydrate recognition site (Lepur et al., 2012; Yang et al., 1998). Hence, they can be inhibited by competing saccharides. With increasing concentration of galectin-3 added to a fixed concentration of glycoprotein, galectin-3 appears to self-aggregate, as the number of bound molecules exceeds the number of carbohydrate binding sites on the target ligand, and saturation of galectin-3 binding is not reached (Lepur et al., 2012; Massa et al., 1993). To explain this, a model called type-C self-association was proposed (see poster). According to this model, the ligand initiates galectin-3 self-association, but is not needed for its extension. Most tested ligands so far are at least bivalent glycoconjugates, but in one case a monovalent tetrasaccharide was suggested to induce type-C self-association (Halimi et al., 2014). Perhaps also non-carbohydrate ligands could be initiators?

The above-mentioned model has some practical consequences. Because the self-association *per se* can be inhibited by lactose, an involvement of carbohydrate ligands is not absolutely necessary. Furthermore, once a ligand induces type-C self-association, it triggers more galectin-3 clustering than expected from its own concentration (Vrasidas et al., 2003; Zhang et al., 2018). Similarly, when galectin-3 or its CRD are added to cells, they will enhance binding of additional galectin-3, as opposed to what is observed with saturable receptors (Lepur et al., 2012; Sundqvist et al., 2018). The propensity for galectin-3 to associate with itself and carry along other proteins also highlights that any interactions detected by co-immunoprecipitation or affinity chromatography of cell extracts need to be interpreted with caution. However, the proposed type-C self-association model does not provide an easy explanation for how cross-linking occurs, and how the N-terminal parts of galectin-3 contribute.

Addition of galectin-3 to a classical binding partner, asialofetuin (ASF), resulted in fairly uniform particles of about 1 µm, as determined by dynamic light scattering (Lepur et al., 2012) and microscopy (H. Leffler, unpublished), suggesting these might be liquid droplets. In support of this, the repeat part of galectin-3 indeed resembles intrinsically disordered regions (IDRs); it also has hydrophobic properties and has been shown to aggregate by itself (Hsu et al., 1992; Lin et al., 2017; Yang et al., 1998).

In another model, galectin-3 is proposed to form defined oligomers, possibly pentamers, based on N-terminal domain association (Ahmad et al., 2004). Here, addition of increased concentrations of a divalent saccharide to a fixed concentration of galectin-3 gave rise to a maximum of precipitate that was formed with a ratio of galectin-3 to saccharide of about 2.5, suggesting the formation of a pentamer (Ahmad et al., 2004a). However, an alternative explanation could be that excess saccharide inhibits type-C self-association, as was found to be the case when an excess of ASF was titrated into a fixed concentration of galectin-3 (Lepur et al., 2012). The reverse experiment, adding galectin-3 into a fixed concentration of the divalent saccharide was not reported. The pentamer model does not explain why saturation is not reached in the latter type of titration experiment. Cross-linking between the Pro-Gly-Tyr-rich repeat regions of galectin-3 might occur (see poster), as these by themselves appear to aggregate (Hsu et al., 1992; Lin et al., 2017). However, as opposed to what is shown in many schematic drawings, no experimental evidence has yet been provided for an association between the 30 aa N-terminal parts of galectin-3.

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Table S1. Main defects in galectin knockout mice

Galectin ko	Phenotype	References
Galectin-1	Viable, few neural progenitor cells, insufficient tumor angiogenesis, impaired B-cell development	(Espeli et al., 2009; Poirier and Robertson, 1993; Sakaguchi et al., 2006; Thijssen et al., 2006)
Galectin-2	Viable, Low level of apoptosis in lymph nodes, decrease in neutrophils, increase in lymphocytes, increased red pulp areas in spleen	(Orr et al., 2013)
Galectin-3	Viable, altered inflammation, reduced fibrosis in many tissues, glomerular injury, increased airway sensitivity against infections, improved insulin sensitivity	(Clare et al., 2014; Colnot et al., 1998b; Henderson and Sethi, 2009; Hsu et al., 2000; Li et al., 2016; Pugliese et al., 2001)
Galectin-7	Viable, defects in the maintenance of epidermal homeostasis, defective wound-healing capacity	(Gendronneau et al., 2008)
Galectin-8	Viable, Reduced VEGF-C-induced lymphangiogenesis	(Chen et al., 2016)
Galectin-9	Viable, Increased antibody production, lymphocytes and neutrophils	(Orr et al., 2013)
Galectin-12	Viable, Increased adipocyte mitochondrial respiration, reduced adiposity, and ameliorated insulin resistance/glucose intolerance	(Yang et al., 2011)
Galectin-1 and -3	Viable	(Colnot et al., 1998a)

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