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Review

How galectins have become multifunctional proteins

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Summary. Having identified glycans of cellular glycoconjugates as versatile molecular messages, their recognition by sugar receptors (lectins) is a fundamental mechanism within the flow of biological information. This type of molecular interplay is increasingly revealed to be involved in a wide range of (patho)physiological processes. To do so, it is a vital prerequisite that a lectin (and its expression) can develop more than a single skill, that is the general ability to bind glycans. By studying the example of vertebrate galectins as a model, a total of five relevant characteristics is disclosed: i) access to intra- and extracellular sites, ii) fine-tuned gene regulation (with evidence for co-regulation of counterreceptors) including the existence of variants due to alternative splicing or single nucleotide polymorphisms, iii) specificity to distinct glycans from the glycome with different molecular meaning, iv) binding capacity also to peptide motifs at different sites on the protein and v) diversity of modular architecture. They combine to endow these lectins with the capacity to serve as multi-purpose tools. Underscoring the arising broad-scale significance of tissue lectins, their numbers in terms of known families and group members have steadily grown by respective research that therefore unveiled a well-stocked toolbox. The generation of a network of (ga)lectins by evolutionary diversification affords the opportunity for additive/synergistic or antagonistic interplay *in situ*, an emerging aspect of (ga)lectin functionality. It warrants close scrutiny. The realization of the enormous potential of combinatorial permutations using the five listed features gives further efforts to understand the rules of functional glycomics/lectinomics a clear direction.

Key words: Adhesion, Glycosylation, Lectin, Proliferation, Sialylation

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Introduction

The ubiquitous presence of glycans has posed the enigma of their functional significance for decades, "a sugary coating" (or glycocalyx, a term introduced in 1963 by H. S. Bennett) being typical for pro- and eukaryotic cells (Gasic and Gasic, 1962; Bennett, 1963; Rambourg et al., 1966; Ito, 1969). Proteins and sphingolipids alike serve as scaffolds to present the glycan chains (Klenk, 1942; Ginsburg and Neufeld, 1969; Kornfeld and Kornfeld, 1976; Stults et al., 1989; Sharon and Lis, 1997; Buddecke, 2009; Corfield, 2015, 2017; Kopitz, 2017). They are the products of a large machinery comprised of more than 500 components that take care of all steps from the synthesis of building blocks and their activation to oligomer assembly and dynamic remodeling (Brockhausen and Schachter, 1997; Habuchi et al., 1998; Reuter and Gabius, 1999; Chen et al., 2001; Kaneko et al., 2001; Patsos and Corfield, 2009; Wilson et al., 2009; Zuber and Roth, 2009; Moremen et al., 2012; Hennet and Cabalzar, 2015; Schengrund, 2015; Bhide and Colley, 2017; Kaltner et al., 2019; Parker and Newstead, 2019; Reily et al., 2019). The term 'complex carbohydrates' for the

resulting wide array of compounds, the glycome of a cell, graphically reflects the exceptionally high level that the natural variability among glycans reaches compared to that of oligonucleotides and peptides (Sharon, 1975).

The initial assumption that strategically surface-positioned carbohydrate oligo- and polymers can be signals for cellular recognition has developed into the paradigm of the sugar code (Eylar, 1965; Roseman, 1970, 2001; Cook, 1986, 1995; Schnaar, 1991; Gabius, 2006, 2009; Lopez and Schnaar, 2009; Kaltner et al., 2018; Kaltner and Gabius, 2019). In fact, carbohydrates are now considered as (third) alphabet of life falling into line with nucleotides and amino acids, each monomer representing a 'letter'. The chemical properties of monosaccharides, most prominently the presence of a hydroxyl group at (nearly) each carbon atom ready for chain building with an activated donor, facilitate to enzymatically generate many more 'code words' than is possible by the 5',3'-phosphodiester linkage between nucleotides or the peptide bond between amino acids (Rüdiger and Gabius, 2009). Glycans as a molecular message hence reach an unsurpassed level of information density in a minimum of space (Winterburn and Phelps, 1972; Laine, 1997; Gabius and Roth, 2017). Noteworthy for turning glycan-encoded information into bioactivity, the ability of each carbohydrate to engage in the directed modes of hydrogen, coordination and C-H/π bonding makes highly specific recognition processes with receptors possible. The site-specific introduction of phosphate and sulfate groups into glycans expands this potential, as is the case by post-translational modifications of proteins with these two groups.

Historically, the selectivity and the specificity of glycan-protein interactions were traced first by detecting activities in plant extracts that agglutinate erythrocytes strictly according to their histo-blood group ABH status. This finding led to coining the term lectin "from Latin *lectus*, the past participle of *legere* meaning to pick, choose or select" or to read in order to distinguish these (glyco)proteins from similarly agglutinating serum antibodies (Boyd, 1954; Boyd and Shapleigh, 1954). As a laboratory tool, lectins soon found widespread applications to spot and to localize carbohydrate epitopes cyto- and histochemically (Roth, 1987, 2011; Taatjes and Roth, 1991; Spicer and Schulte, 1992; Danguy et al., 1994; Manning et al., 2017a). This binding can not only establish a firm contact between cells but can also cause outside-in signaling, thereby triggering cellular responses such as mitogenesis or apoptosis (please see below).

The capacity of plant lectins (phytohaemagglutinins) to 'read' glycan-encoded 'messages' and then to 'translate' them into post-binding responses was first described for the so-called phytohaemagglutinin (PHA; from red kidney beans (*Phaseolus vulgaris*)). It "was found to be a specific initiator of mitotic activity" in leukocytes of heparinized whole blood (Nowell, 1960). This finding had paradigmatic importance for the search of the missing link between a cellular glycoconjugate and glycan-inhibitable processes such as cell adhesion or control of proliferation in animals and man. The following verbatim quotation puts the underlying hypothesis (expectation) into words: since "it has been suggested that complex carbohydrate-containing molecules may function in synaptic recognition and transmission through establishment of cell-cell contacts and possibly also as mediators of communication between the surface and the interior of the cell", "the presence in neural tissue of enzymes and proteins capable of interacting with saccharides is to be expected". Indeed, by performing the respective experiments (i.e. haemagglutination assays with trypsinized rabbit erythrocytes to detect lectin activity in extracts and affinity chromatography on resin presenting galactose as ligand to purify the active protein from organ extract), this assumption was proven to be true: a β-galactoside-binding protein with strong agglutinating activity was isolated at a yield of about 400 mg/kg from electric organ tissue of *Electrophorus electricus* (Teichberg et al., 1975). Moreover, agglutinating activity blocked by β-galactosides such as thiodigalactoside was shown to be present in various tested mammalian and avian organs and cells.

Measuring high titers of thiodigalactoside- and lactose-inhibitable haemagglutination with extracts of chick embryo (pectoral muscle) and of a murine neuroblastoma (N-18) cell line, less so in extracts of adult rat organs, pointed to a widespread presence and pronounced developmental regulation of corresponding protein(s) (Teichberg et al., 1975). One way to meet the expectation for a broad-scale occurrence of tissue constituents with this binding capacity, matching the ubiquitous presence of glycans, was taken by applying a special technique called glycocyto- and histochemistry: β-galactosides as the ligand part of labeled (neo)glycoconjugates detect the presence of sugar receptors in cells and in tissue sections and thus are an efficient tool for screening. Specific (glycan-inhibitable) positivity was obtained during monitoring for binding with these custom-made synthetic reagents in diverse organs and cell types (for examples, please see Gabius et al., 1987, 1988, 1990; Sinowatz et al., 1988; Bardosi et al., 1989; for review, please see Gabius et al., 1993). Illustrating a possible clinical relevance, the presence of binding sites for histo-blood group A and H epitopes (both known high-affinity ligands for lectins) in sections of lung tumors correlated positively with survival of cancer patients (Kayser et al., 1994). Stepwise elaboration of the structural complexity of the glycan part of neoglycoconjugates up to complex-type N-glycans and glycodendrimers with multiple branching is feasible by elegant chemical and chemoenzymatic protocols (Unverzagt et al., 2002; Chabre and Roy, 2009, 2010; Oscarson, 2009).

These pioneering findings on widespread occurrence of lectin activity in extracts, cells and tissue sections prompted to proceed to purify the detected galactose-binding lectins by affinity chromatography and to

determine their characteristics; most important for a classification of the proteins is the sequence. The more sequences became available, the clearer the notion became for the existence of not just very few proteins with this specificity but of a family. By performing calculations of the homology score of their sequences, a phylogenetic relationship was unveiled, and the term S-type lectins (due to the requirement to add a thiol such as dithiothreitol or β-mercaptoethanol to protect the activity for haemagglutination of at that time known family members) was coined, in analogy to 'C-type' for a group of homologous lectins that require Ca^{2+} for lectin activity (Drickamer, 1988). Interestingly, the first member of this family traced by its endocytic activity on hepatocytes for an asialoglycoprotein ("exposed, terminal galactosyl residues were identified as specific determinant for hepatic recognition and uptake") and thus originally called asialoglycoprotein receptor shares specificity to this type of sugar (Morell et al., 1968, 1971; Hudgin et al., 1974). Today, this family of animal (S-type) sugar receptors is known by the name 'galectin', and research on their structural and functional aspects is a burgeoning area (Harrison and Chesterton, 1980; Barondes, 1984, 1997; Kasai and Hirabayashi, 1996; Gabius, 1997; Hirabayashi, 1997, 2018; Cooper, 2002; Kaltner and Gabius, 2012; Kaltner et al., 2017; de Jong et al., 2020). The next section explains in detail the currently accepted criteria for calling a protein 'galectin'.

What is a galectin?

Firstly, a galectin is classified as a lectin, meaning that it is able to bind carbohydrates without being an antibody, an enzyme that processes the bound sugar or a sensor/transporter for free mono- to oligosaccharides (Kocourek, 1986; Barondes, 1988; Gabius et al., 2011). Considering that the already mentioned disaccharides with non-reducing-end galactose, i.e. thiodigalactoside and lactose (Lac), are the canonical inhibitors of galectin-dependent haemagglutination, a simple shortening of the resulting term 'galactose-binding lectin' by four syllables explains the origin of the now commonly used term ga(lactose-binding)lectins. Since specificity to β-galactosides does not suffice for an unambiguous classification, other types of lectins such as mentioned C-type lectins, the biohazard ricin or bacterial and fungal adhesins, for example during uropathogenic colonization (Ielasi et al., 2016; Moonens and Remaut, 2017), also binding galactose, two structural properties are added to reach a clear separation. They are the three-dimensional arrangement to give stability to the binding site and characteristic sequence features within the contact region. The carbohydrate recognition domain (CRD) of a galectin is a β-sandwich (Fig. 1). In this type of fold, a set of amino acids called the signature sequence is responsible for contact building to the ligand, and this is why they are mostly conserved (Fig. 1). These two structural features, i.e. global fold and sequence signature, complete the galectin definition.

That this 3-point definition, i.e. mono- or disaccharide specificity, fold and sequence signature, is vital is further highlighted by noting that more than a dozen types of protein domains have acquired the capacity to accommodate glycans as ligands, among them the β-sandwich. Deserving particular attention, galectins are not the only β-sandwich-type lectins: four other classes of mammalian lectins use this fold as a platform, i.e. the pentraxins, the endoplasmic reticulum

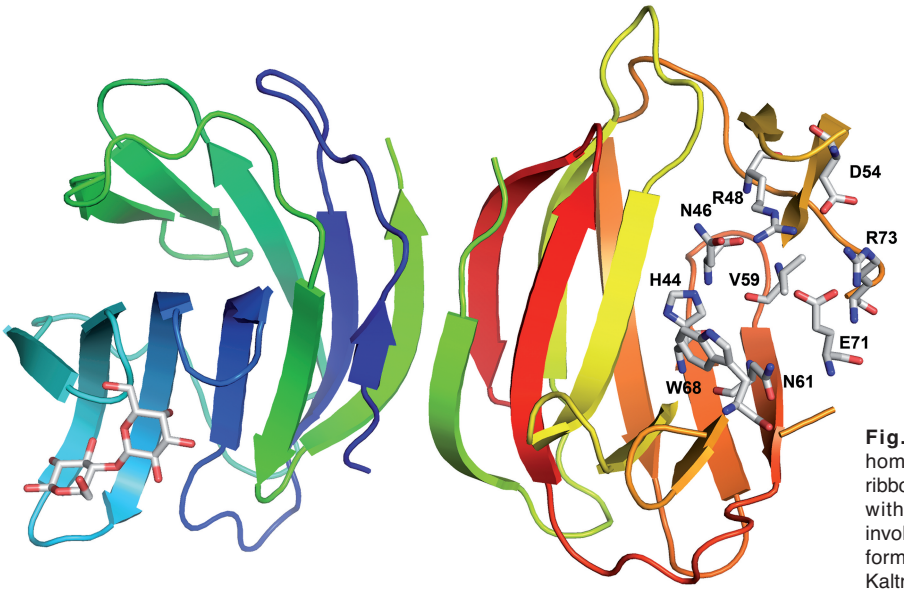


Fig. 1. Illustration of the crystal structure of homodimeric chicken galectin (CG)-1A (1QMJ) as ribbon diagram (left: with bound ligand lactose; right: with structures of highly conserved amino acids involved in binding cognate β-galactosides and thus forming the sequence signature for galectins; from Kaltner and Gabius, 2012, with permission).

(ER)-based chaperones calnexin and calreticulin, the cargo handlers between ER and Golgi apparatus such as ERGIC53 and the CRD of N-glycanase, also called PNGase (Fujimoto et al., 2014; Solís et al., 2015). Thus, the combination of fold and sequence signature is obviously mandatory for correct and unambiguous assignment of a carbohydrate-binding protein to a lectin family. When relating structures to functions, the apparent development of a large toolbox of lectin families ensures that functional pairing between glycans and lectins (based on complementarity as is the case between strands of nucleic acids or between proteins and nucleic acids in the workings of the genetic code) is likely to reach the degree of molecular diversity that is required for the significance that the term 'sugar code' implies. Toward the same end, developing diversity from an ancestral gene can be expected.

Mechanistically, gene duplications and implementation of sequence divergence during evolution underlie the growth in numbers of members of a family. This is a common route to diversity that has generated the largest group size in the case of the C-type lectins (Gready and Zelensky, 2009; Mayer et al., 2017). The study of this type of CRD has taught the salient lesson that it can gain remarkable versatility in ligand recognition, making Ca^{2+}-independent glycan binding and association to proteins possible, as for example documented for the mannose receptor group and the myeloid C-type lectin receptors (East and Isacke, 2012; Tang et al., 2019). The named mechanisms have similarly been productive in the case of galectins, as illustrated in Fig. 2 by presenting the galectin inventory of several animals from various branches of the phylogenetic tree. Overall, phyla especially rich in galectins are Nematoda, Arthropoda and Chordata (Takeuchi, 2018). Up to 17 different proteins have been identified to be expressed in vertebrates (Fig. 2). Among them are the products of three genes, in which even deviations from the canonical sequence signature are present, i.e. the Charcot-Leyden crystal protein (galectin-10), the eye lens-specific galectin-related inter-fiber protein (GRIFIN) and the galectin-related protein (GRP) (Ackerman et al., 1993; Leonidas et al., 1995; Ogden et al., 1998; Zhang et al., 2000; Cooper, 2002; García Caballero et al., 2016a,b, 2018; Manning et al., 2018a; Ruiz et al., 2018; Ueki et al., 2019). Of note, the occurrence of site-specific changes in the sequence signature can either be confined to distinct species (for GRIFIN; García Caballero et al., 2018) or can generally be found among vertebrates (for GRP; Manning et al., 2018a), and then impairment of binding the canonical glycan ligand ensues. By convention, they are counted among galectins due to the strict conservation of the fold and an overall sequence homology. The structural platform of galectins, as is also known from C-type lectins and closely related C-type lectin-like proteins (Gready and Zelensky, 2009), thus allows for the generation of variants without activity as β-galactoside-specific lectin, a noteworthy hint toward galectin activities beyond requirement for glycan binding. That the galectin CRD can gain additional binding capacity to distinct proteins (please see below) takes the analogy even one step further.

In summary, the classical definition states that a galectin is a β-galactoside-binding protein with β-sandwich fold and conserved sequence signature. Phylogenetically, the development of galectins into a family began with an ancient ancestor. It most likely had the (precursor of the) sequence signature for facilitating contact formation with β-galactosides. Vertebrates express two proteins that belong to this family despite the presence of deviations from this typical signature, i.e. GRIFIN and GRP. The ubiquitous presence of galectins in the animal kingdom illustrated in Fig. 2 very much suggests fundamental role(s), likely by an interplay with omnipresent galactose-containing glycans. Investigations on galectin activities that started with the described detection of the ability to bridge erythrocytes have opened a very fertile research area rich in discoveries. They have led to the conclusion that an initially assumed "one galectin – one phenomenon relationship does not exist" (Kasai, 1997). In contrast, galectins are now considered to be multifunctional. What does this mean?

The multifunctionality of galectins

In principle, the term 'gene' had often been associated with the expectation that its product has a single function. "However, this orderly view of biology has turned out to be overly simplistic", one reason resting on the discovery of moonlighting (Copley, 2012). That eye lens crystallins and enzymes of metabolic pathways or cycles (argininosuccinate lyase, α-enolase and lactate dehydrogenase) or the neurotrophic factor neuroleukin and phosphohexose isomerase were revealed to be identical ("sharing genes"; Piatigorsky et al., 1988) is by no means exceptional. In fact, an increasing number of proteins have been and are being described to exert more than one autonomous activity by distinct sites of a domain (Jeffery, 2003, 2009, 2018; Huberts and van der Klei, 2010; Copley, 2012; Zanzoni et al., 2019), and galectins are among them. In addition, and in fact providing a further source for multifunctionality, galectins can cause pleiotropic effects due to functional pairing with counterreceptors such as cognate glycans of different glycoproteins via the same contact site, namely the one for β-galactosides shown in Fig. 1, and downstream signaling along various pathways (please see below). Multifunctionality of galectins can arise, too, by structural variations based on single nucleotide polymorphism, alternative splicing, shifts to different types of architecture to present the CRD and the modular design of a protein when it consists of more structural units than the CRD (please see below). Their ability to aggregate cells in a carbohydrate-inhibitable manner is therefore just one, a readily measurable aspect of lectin functionality. After all, the binding of a lectin to a cell surface can do (much)

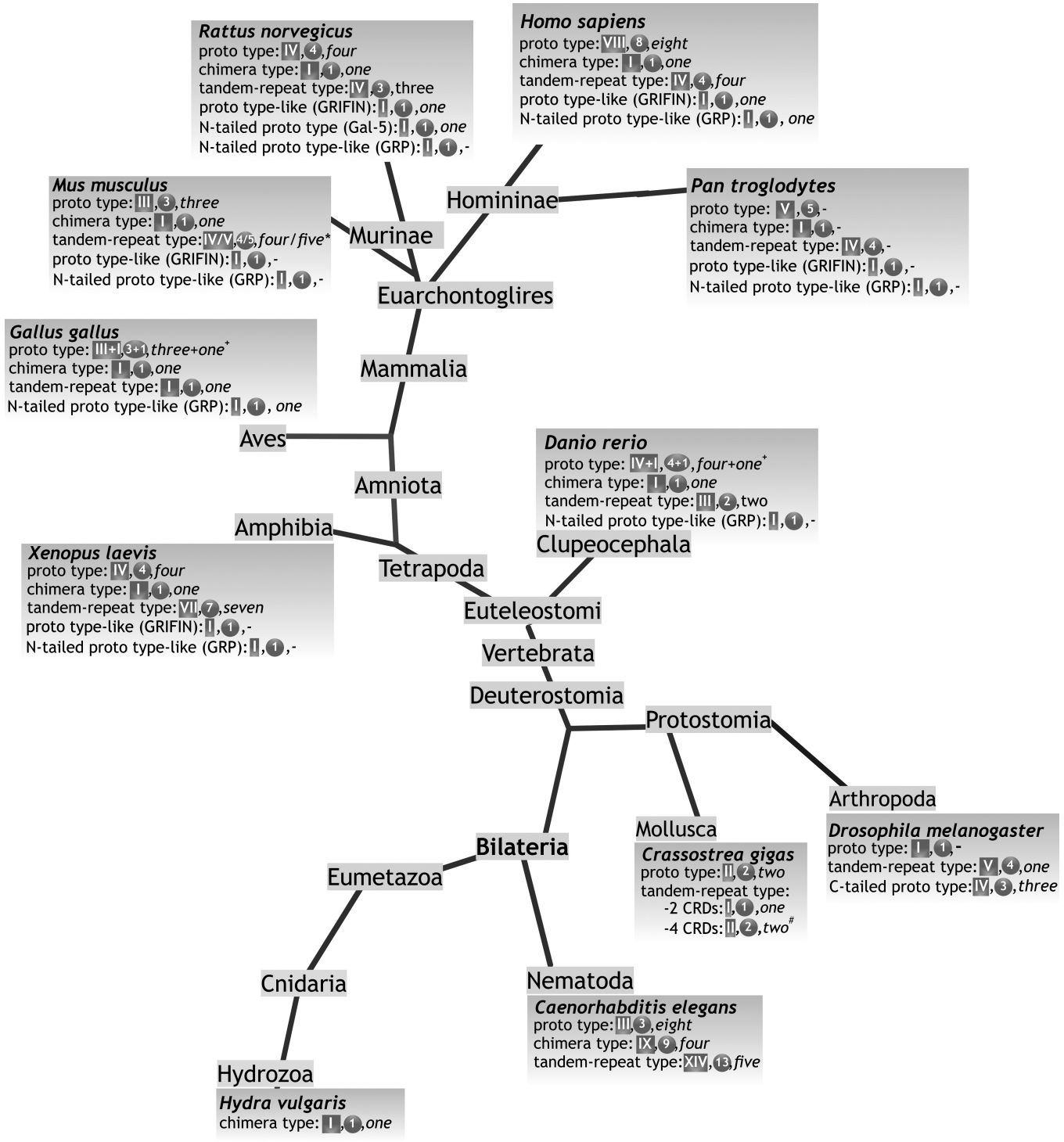


Fig. 2. Illustration of the composition of the galectin family in selected organisms at different positions of the phylogenetic tree (for schematic presentation of the main types of galectin architecture, please see Fig. 8). Evidence for presence of galectins on the level of the gene (Roman number), the mRNA (Arabic number) and the protein (numerical denotation) is summarized and given as number in each case. Special cases are highlighted: GRIFIN with its species-dependent variability of lectin activity (+) and the presence/absence polymorphism of Gal-6 exclusively found in certain mouse strains (*) as well as the tetrameric design of two distinct oyster galectins (#); from Kaltner and Gabius, 2012, updated and extended.

more than to let cells make contacts.

The first evidence for a cellular response following surface binding of an animal lectin came from testing for an effect on proliferation, in analogy to the already noted case of PHA that initiated to trace the now widely documented mitogenicity of plant agglutinins (Nowell, 1960; Borrebaeck and Carlsson, 1989). Murine Thy-1-negative spleen cells and lymphocytes but not chicken thymocytes were stimulated by homodimeric chicken galectins (Lipsick et al., 1980; Pitts and Yang, 1981; Levi and Teichberg, 1985). The rate of proliferation was also found to be enhanced by mammalian galectins in cell types different from immune cells, first in the case of bovine pulmonary artery endothelial cells and smooth muscle cells (Sanford and Harris-Hooker, 1990) and thereafter also for example in the case of human lung (IMR-90) fibroblasts (Inohara et al., 1998). Remarkably, at the same time, proteins referred to as "transforming growth factor (TGF)-γ_2" (due to functional similarity to TGF-α/β; Yamaoka et al., 1991) or as "autocrine negative growth factor" (causing S/G_2 growth arrest, in leukemic cells followed by apoptosis (likely via inducing predominance of Bax over Bcl-2; Novelli et al., 1999)) were found to have the sequence of a galectin, i.e. galectin-1 (Gal-1): they yet did not require sugar binding for activity (Wells and Mallucci, 1991; Yamaoka et al., 1993). This galectin's growth-regulatory activity profile is induced by outside-in signaling via various pathways following glycan- or protein-mediated binding; it covers the range from mitogenicity to growth arrest, also including anoikis/apoptosis. The actual outcome depends on the cell type as well as its functional and phenotypic status (for reviews, please see Pace and Baum, 1997; Hsu and Liu, 2002; Yang and Liu, 2003; Villalobo et al., 2006; Smetana et al., 2013). As a consequence, Gal-1 has been called "a bifunctional regulator of cellular proliferation", and, "unsurprisingly, the interpretation of the actions of Gal-1 in developmental situations, both normal and neoplastic, is often very complex" (Scott and Weinberg, 2004).

What inspecting the effects of Gal-1 on cell growth teaches us is that the same protein called galectin can exert entirely different effects on a characteristic of a cell, and this by pairing with various molecular targets and/or post-binding routes. Dual (Janus-like) effects are triggered beyond this special functional aspect. Concerning adhesion, tipping the balance in either direction is possible: whether cell attachment to a matrix, e.g. to glycoproteins such as fibronectin or laminin, or aggregate formation will be favored or blocked is a matter of the properties of the experimental setting (for examples on Gal-1, -3 and -8, please see Cooper et al., 1991; André et al., 1999; Levy et al., 2001; Friedel et al., 2016). By proceeding to test galectins in diverse assay systems and analyzing their interaction with counterreceptors, the activity profile was extended step by step. At present, the already known range of functional versatility of galectins, compiled in Table 1, is broad, and this table makes obvious why galectins have been described to present more than one face to the observer (Kasai, 2018).

When looking at Table 1, the collective body of evidence that underlies this summary justifies to call galectins multifunctional proteins - and it raises the question how it can be explained that they succeeded in becoming multitalented proteins or colloquially "jack of all trades" (a term applied explicitly to Gal-3; Newlaczyl and Yu, 2011). The following five sections answer this question by focusing on the corresponding features of galectin genes and proteins as well as describing their up to now delineated contributions to the galectins' functional complexity. The first section on the absence of the sequence for a signal peptide solves the mystery of why galectins are generally found intracellularly. This aspect of localization engenders the opportunity for a dedication of these effectors to function in cells.

Table 1. Survey of functions of vertebrate galectins[a].

Intracellular	Cell surface/extracellular
	bridging cells
nuclear	bridging cells and matrix
involved in pre-mRNA splicing	bridging (glyco)proteins
regulating gene expression	contributing to host defence and tissue remodeling (cell (e.g. mast cell or neutrophil) activation, opsonization, scavenging, sensing foreign glycans)
	eliciting outside-in signaling
	- cell death/growth regulation
cytoplasmic	- gene expression
regulating cell death/growth	- mediator production and release
routing glycoproteins	regulating
sensing damage to intracellular vesicles and calling for help	- cell migration/motility
	- surface residency of glycoproteins by cross-linking slowing endocytosis

[a]type of activity depends on spatiotemporal context and properties of respective counterreceptor in the particular context, with potential for triggering either negative or positive effects (e.g. anti- or pro-adhesive/inflammatory); (patho)physiological read-out in each aspect is often complex with noted specificity for cell type (e.g. diverse classes of mediators such as chemo- and cytokines, enzymes such as MMPs or reactive oxygen species and distinct members for each class)

Toward multifunctionality: absence of a signal peptide

This property commonly accounts for protein presence in intracellular compartments (Arnoys and Wang, 2007), and it makes vertebrate galectins special among lectins of these species. Proof-of-principle studies on Gal-1 have revealed the synthesis of galectins on free ribosomes (Wilson et al., 1989). As a consequence, galectins, when produced, are cytoplasmic proteins, also capable of entering the nucleus (Smetana et al., 2006; Haudek et al., 2010; Funasaka et al., 2014), this pattern of immunohistochemical positivity being exemplarily shown in different types of cells/organs in Fig. 3a-d. Panel d of this figure already indicates disparity in distribution of staining for galectins. The discovery of export of Gal-1 from the cytosol to the extracellular space, first described in the case of murine muscle (C2) cells by its localization, actually together with laminin extracellularly, and by immunoprecipitation of externalized protein after metabolic labeling (Cooper and Barondes, 1990), was more than just a reason to let galectins join the rather small class of proteins that are secreted via non-classical routes. Interestingly, pioneering studies on exploring the possibility for galectin externalization had been done on chicken pectoral muscle and intestine (Barondes and Haywood-Reid, 1981; Beyer and Barondes, 1982).

Looking at the biochemical nature of other proteins traveling from the nucleus and/or the cytoplasm to the extracellular space, "many of the known unconventional secretory proteins are cytokines, growth factors, or other molecules with important signaling roles in physiological processes such as inflammation, angiogenesis, cell differentiation, or proliferation" (Nickel and Seedorf, 2008). Important for alerting and orchestrating defence (and even "more than just signaling the alarm"; Roan et al., 2019), extracellular availability of endogenous effectors of this group contributes to the so-called damage-associated molecular patterns (DAMP), metaphorically referred to as (dual-function) alarmins (Matzinger, 1994; Oppenheim and Yang, 2005; Bianchi, 2007; Bertheloot and Latz, 2017; Yang et al., 2017), and galectins (especially Gal-3) have been nominated as candidates to join this group under certain circumstances (Sato and Nieminen, 2002; Sato et al., 2009). To become a member of this group points to an equally eminent physiological relevance for galectins, and this at different sites of a cell.

Following the demonstration of translation on free ribosomes, galectin routing through the ER and Golgi apparatus was independently excluded. Experimentally, no impact on extent of secretion of another galectin, i.e. Gal-3, was measured when blocking this classical export route via the ER and Golgi apparatus by brefeldin A (Lindstedt et al., 1993; Sato et al., 1993). Galectins thus find their way to the extracellular space (matrix, cell surface or medium), hereby residing in- and outside of cells, and become an active part of communication between cells. "The presence of galectin-1 in extracellular matrix [(ECM)] affects interactions of cells with ECM, such as cell adhesion, cell migration, and cell survival" (He and Baum, 2006), as already noted above. Precluding autoimmune disease onset for example by bridling effector T cell activity or driving tumor progression are clinically relevant examples for the two sides of the coin of this type of auto- and/or paracrine communication (Wang et al., 2009; Wu et al., 2011; Smetana et al., 2015; Orozco et al., 2018). The dual localization in- and outside of cells lets galectins engage in contacts at various sites of cells, all this made possible by the absence of a signal sequence.

Concerning the molecular basis of their export, it is being revealed that crossing the membrane by vesicular and non-vesicular transport is facilitated by a steadily growing number of non-conventional mechanisms (Hughes, 1999; Popa et al., 2018; Sato, 2018; for an instructive illustration, please see Fig. 3 in Sato, 2018). Definitely, galectin export is not a random process. As informative as the ongoing dissection of these externalization processes is, this work has recently been flanked by an alternative strategy: answering the question on what will happen if a galectin is instructed to enter the ER by protein engineering, that is by adding a leader sequence to its cDNA, will help us understand better why galectins lack a signal sequence. Interestingly, several human galectins have an N-glycosylation motif (Kutzner et al., 2020) so that they might become glycoproteins.

When pursuing this approach by combining cDNA engineering and ectopic expression, conventionally secreted galectin protein could be obtained from yeast cells and its properties analyzed. Maintained activity of lactose binding of the product as well as transfer of an N-glycan to the sequon of human Gal-1 at ^{95}NLT^{97} and its processing were documented, also glycosylation and secretion of Gal-4 from human embryonic kidney (HEK293T) cells (Kutzner et al., 2020). These results suggest the following conclusion: by taking unconventional export routes, a galectin literally avoids becoming N-glycosylated at available sequon(s). After all, the presence of an N-glycan on human Gal-1 impacts galectin function. It has been shown to reduce both the lectin's affinity and growth-regulatory activity elicited by counterreceptor (ganglioside GM1) cross-linking on the surface of human neuroblastoma (SK-N-MC) cells, likely by interfering with counterreceptor binding and/or appropriate lattice formation (Kutzner et al., 2020); and an N-glycan will also cover a sizeable region on the protein's surface, thereby masking it and blocking formation of respective contacts (please see below).

In summary, the absence of a signal peptide ensures production of active (non-glycosylated) protein, which will then first be present in the cytoplasm and then also in the nucleus; the development of various mechanisms for externalization ensures that the protein can reach the extracellular compartment. A constitutive expression as a house-keeping activity would render galectins unable

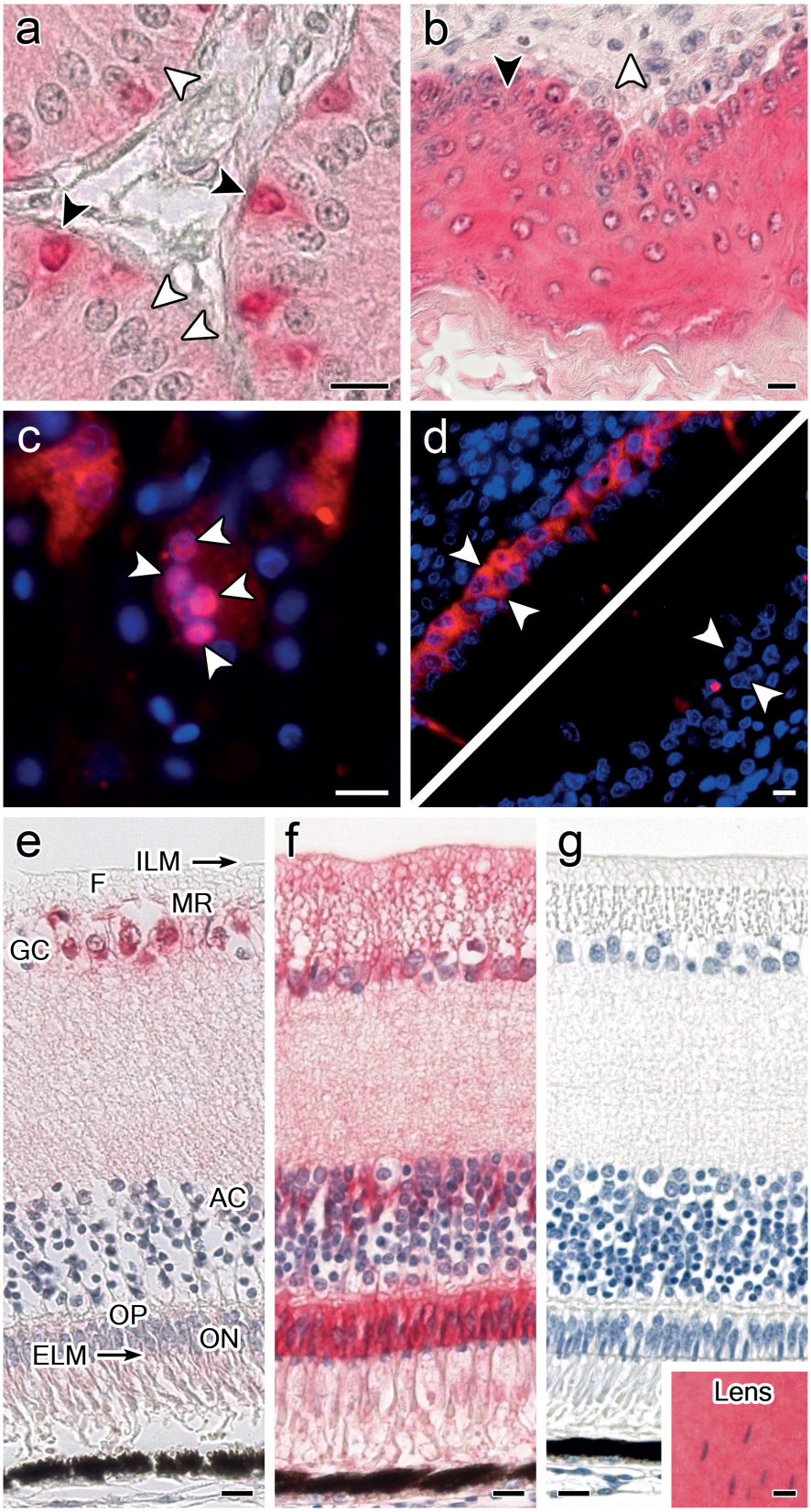


Fig. 3. Illustration of immunohistochemical detection of galectins in different cell types and subcellular compartments by light (**a, b, e-g**) and fluorescence microscopy (**c, d**). Antibody binding was visualized either with alkaline phosphatase-conjugated second-step reagents and Vector® Red AP substrate or by Alexa Fluor® 568-labeled second-step antibody. **a.** In fixed murine initial-segment epididymis, Gal-3-specific staining was seen in nuclei of basal cells (black arrowheads) in contrast to only, if at all, weak staining of the cytoplasm and of nuclei of principal cells (white arrowheads). **b.** In esophagus of the adult rat, Gal-7 was detected in the cytoplasm of the non-keratinized stratified squamous mucosal epithelium (black arrowhead). The underlying lamina propria was negative (white arrowhead). **c.** In fixed developing chicken bone, signal for CG-3 (nominally red) in the nuclei (arrowheads) of osteoclasts overlaps with the 4',6-diamidino-2-phenylindole (DAPI) signals for counterstained nuclei and thus appears in magenta. **d.** CG-3 was detected in a subnuclear and perinuclear band-like area within the pseudostratified columnar epithelium of adult chicken ureter (white arrowheads, top left), whereas the respective area was negative for CG-1A (white arrowheads, bottom right). In sections of fixed adult chicken retina (**e-g**), qualitative and quantitative differences in CG expression were documented for: CG-2 was almost exclusively seen in the ganglion cell layer (GC) (**e**), CG-3 was strongly expressed in the inner layers including the GC, roots of Müller cells (MR), nerve fiber layer (F) and internal limiting membrane (ILM), as well as also prominently in the amacrine cell layer (AC), the outer plexiform (OP) and the outer nuclear layer (ON) up to the external limiting membrane (ELM) (**f**). **g.** In contrast, the retina was completely negative for C-GRIFIN expression, which is exclusively found in the lens (inset of g). Lack of staining in retina also serves as negative control. Antibody concentrations: anti-CG-1A: 2 μg/mL; anti-CG-2: 2 μg/mL; anti-CG-3: 0.125 μg/mL (d) or 0.5 μg/mL (c,f); anti-Gal-3 (murine): 0.5 μg/mL; anti-C-GRIFIN: 2 μg/mL. Scale bars: 10 μm.

to participate effectively in cellular dynamics and stage-specific processes among cell types. A galectin therefore has access to binding partners both intra- and extracellularly. In order to achieve spatiotemporally regulated expression, for example to switch production on or off during differentiation or a pathogenetic process, galectin genes are expected to exhibit a distinct feature, that is a promoter region equipped with a large number of motifs for binding transcription factors so that combinatorial permutations are plentiful toward the given aim.

Toward multifunctionality: presence of fine-tuned gene regulation

This trait of intimately controlled galectin expression already became visible when measuring galectin activity in extracts of organ specimens obtained in the course of development and finding marked differences at different time points (Den et al., 1976; Nowak et al., 1976; Kobiler and Barondes, 1977) as well as when starting to explore their expression profiles cyto- and immunohistochemically, e.g. by monoclonal antibodies against the Mac-2 antigen (Gal-3). In murine lung, to give an example, alveolar macrophages and bronchial epithelium were positive, and a strong response to "specific differentiative signals" was found by measuring pronounced upregulation of percentage of positivity of peritoneal macrophages (from <2% to >96%) upon i. p. injection of thioglycollate into mice to obtain peritoneal exudates (Ho and Springer, 1982; Flotte et al., 1983). Initial mapping of the promoter region of the human Gal-3 gene pinpointed the presence of multiple sites with putative affinity for transcription factors spread all over this sequence, this study also providing initial evidence for respective relationships to explain why serum addition can turn on Gal-3 production (Kadrofske et al., 1998). Similarly, the upregulation of Gal-1 expression by butyrate was attributed to a distinct sequence motif defined as an Sp1-binding site (at -57 to -50; GGGGCGGG) in the 5'-proximal promoter region of the gene for human Gal-1 (Lu and Lotan, 1999). The ensuing detection of a variability in increasing gene activation by butyrate among human galectins attests to the existence of promoter divergence (Katzenmaier et al., 2014). Indeed, these regions of galectin genes evidently contain the postulated sets of sequence motifs for effective regulation, leading to "finely tuned" expression (Chiariotti et al., 2004).

In order to illustrate the occurrence of multiple sequence motifs and distinctive patterns for potential of binding transcription factors among promoter regions of human galectin genes, an overview of the results of the comparison between respective sequence hits for Gal-1, -3 and -8 is presented in Fig. 4, separated into unique and shared features. Obviously, the molecular set-up is available to facilitate instances of stand-alone presence or of co-expression of galectins, giving direction to the detailed functional promoter analysis. In addition to the possibilities for intricate regulation at this level, transcription of galectin genes can start from different sites, products then being subject to alternative splicing and generation of various polyadenylation sites (for information on examples of Gal-3 and -8, please see Voss et al., 1994; Gopalkrishnan et al., 2000; Bidon et al., 2001; Kaltner et al., 2009, 2011).

In aggregate, the inspection of untranslated gene sequences and the current status of their functional analysis raise the expectation for differential expression of galectins, in time (e.g. embryogenesis) and in space (e.g. different cell populations and types). What systematic immunohistochemical monitoring of galectins demonstrates, for example presented in the cases of human Gal-2 and -8 in this journal (Danguy et al., 2001; Saal et al., 2005), is that expression patterns for galectins exhibit cell-type selectivity, up to the unique case of GRIFIN. It is exclusively expressed in the eye lens (Fig. 3g illustrates its absence in retina together with eye lens positivity as positive control), and this under the control of two transcription factors (i.e. L-Maf, Pax-6) that also drive the expression of crystallins (García Caballero et al., 2016b, 2019). That bovine and human α-crystallin interacts with murine GRIFIN (Barton et al., 2009) and that α-, β- and δ-crystallins are among the list of binding partners of chicken GRIFIN (García Caballero et al., 2019, 2020) is suggestive evidence for a concerted expression. Their co-regulation, ability to associate and presence in the main lens body most likely help to establish the tight packing of proteins in the eye lens essential to let it become "biological glass" (Bassnett et al., 2011).

Having herewith described patterns of distribution for individual galectins, the next step is the extension of applying immunohistochemistry to reach a comprehensive analysis for all galectins of an organism, and this requires non-cross-reactive antibodies. Such full-scale monitoring for a vertebrate can most conveniently be attained for a species with a comparatively small number of family members. The respective survey given in Fig. 2 pinpoints chicken as most suited study object with its seven galectins (CGs), which represent all types of vertebrate galectin architecture (details in this structural aspect will be presented below). By taking the mapping of profiles of tissue distribution from single members to the complete family for this test case, the occurrence of individual patterns of distribution with overlaps but also differences were ascertained (Kaltner et al., 2015; Manning et al., 2017b, 2018b). This result is exemplarily documented for adult chicken retina in Fig. 3e-g (for information on galectin mapping in murine organs, please see Lohr et al., 2007 for proto-type galectins and Nio-Kobayashi, 2018 for a review).

That intricate regulatory mechanisms are operative to control expression for each galectin could best be demonstrated in an experimental setting, when the galectin fingerprint is successively taken during the

stages of cellular maturation that starts from a single progenitor cell type. To do so, the eye lens with its origin from a single type of progenitor cell that develops into cuboidal epithelium and highly specialized fiber cells offers a very attractive model (Maisel et al., 1981; Piatigorsky, 1981; Chow and Lang, 2001; Bassnett and Sikic, 2017). The course of cell maturation from the progenitor cells of ectodermal origin to the fully differentiated phenotypes is schematically illustrated in Fig. 5. By analyzing sections from different regions of the eye lens by immunohistochemistry at a distinct stage of development (i.e. HH stage 36) with non-cross-reactive anti-CG-specific antibody preparations, the concept of a fine-tuned regulation given above was verified (Fig. 6; for data on immunohistochemical monitoring during the course of development on the level of the complete CG family, please see García Caballero et al., 2020).

Moving from an application in embryology to histopathology, fingerprinting of galectins in tumors has potential to relate their expression to prognosis and the tumor microenvironment as well as to refine tumor diagnosis (Gabius et al., 1984, 1986a,b; Nagy et al., 2003; Langbein et al., 2007; Saussez et al., 2010; Remmelink et al., 2011; Dawson et al., 2013; Smetana et al., 2015; Živicová et al., 2017, 2018). The detection of association of altered extent of galectin presence with clinical parameters will then give direction to functional assays, as was similarly the case to identify galectins as factors in the pathogenesis of osteoarthritis (Toegel et al., 2014, 2016; Weinmann et al., 2016, 2018).

In summary, lectin expression is subject to an exquisite control that guarantees individual profiles in space and time, far from a uniform pattern of house-

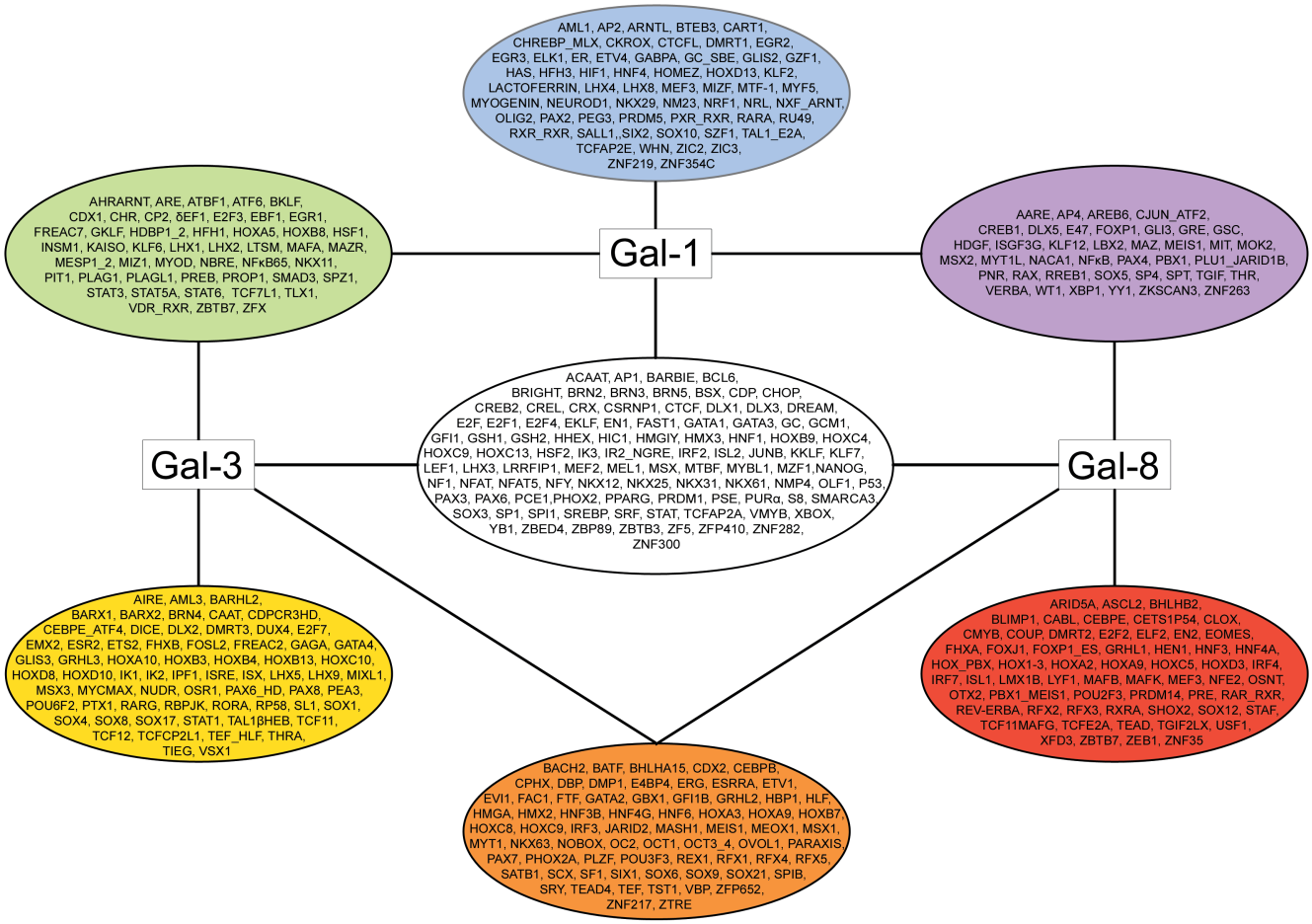


Fig. 4. Illustration of the degree of complexity of putative possibilities for transcriptional regulation of expression of human Gal-1, -3 and -8 genes. Based on computational searches for sequences with predictable affinity for transcription factors in the 2500 bp promoter region upstream of the transcription start site (settings: complete identity of core sequence and entire motif similarity of at least 0.8), respective proteins with at least one potential site for binding are listed (for details on position of sequences and explanation of the acronyms of the identified proteins, please see Toegel et al., 2016; Weinmann et al., 2016, 2018). Sequence motifs for binding can either be unique for a certain galectin (given in blue, yellow and red), shared by a set of two galectins (given in green, magenta or brown) or common for all three galectins (given in white).

keeping proteins. The sophistication of gene regulation together with the access to intra- and extracellular sites by cytoplasmic production and subsequent externalization team up to make a galectin available at distinct places at a certain time in order to engage in functional pairing(s). Looking at the similar degree of plasticity at the level of glycan display determined by (plant/fungal) lectin histochemistry, the notion for rather widespread presence of β-galactosides and a co-regulation of galectins and their counterreceptors is arising. Remarkably, the mentioned case of Gal-1 that has been called a "bifunctional regulator of cellular proliferation" (Scott and Weinberg, 2004) has already indicated versatility with respect to the biochemical nature of ligands. It can be a glycan of a cellular glycoconjugate and it can also be a protein. Thus, the next two sections deal with ligands of galectins.

Toward multifunctionality: glycans as ligands

By the definition for a lectin given above, the canonical (glycan) ligand for a galectin is a β-galactoside. The 'letter' galactose (of the sugar alphabet) is a rather common constituent of the spatially accessible terminal section of N- and O-glycans, of the glycosaminoglycan keratan sulfate and of glycan chains of sphingolipids. Structural variability among β-galactosides is generated by different linkages of galactose to the glycan chain (β1,3 or β1,4; α1,3 in chain extensions), by different subterminal moieties in the chain and different substitutions (sialylation/sulfation, blood group epitopes). As a consequence, a large set of potential ligands for galectins is available (Fig. 7). This situation is clearly different from the presence of a highly unique signal as mannose-6-phosphate is. It is produced in a 2-step synthesis on high-mannose- or hybrid-type N-glycans of a small set of target proteins and is recognized as a postal code for delivery to lysosomes by the two P-type lectins (Dahms and Hancock, 2002; Kornfeld, 2018).

In contrast, most of the glycan chains of vertebrate glycoproteins are known to present β-galactosides. The

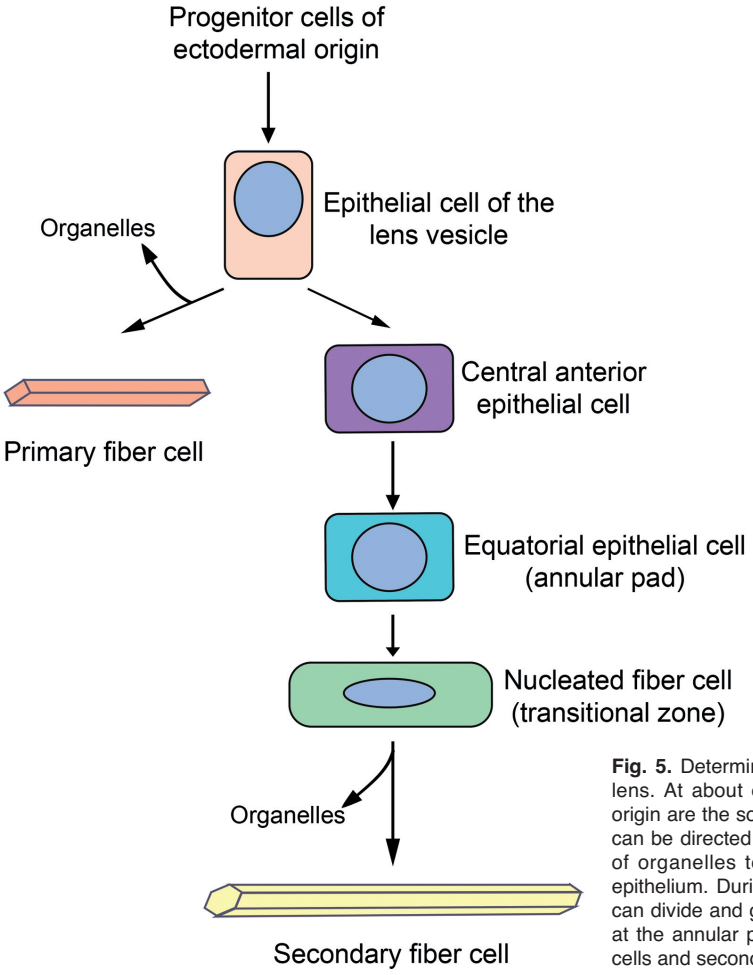


Fig. 5. Determination and differentiation route of cells that form the mature eye lens. At about embryonic developmental stage HH 17-18, cells of ectodermal origin are the source of the epithelium of the lens vesicle. In the next phase, cells can be directed to two routes: to undergo differentiation and elongation with loss of organelles to become primary lens fibers, or to form the central anterior epithelium. During eye lens development, cells in the central anterior epithelium can divide and generate new epithelial cells or can migrate to the equatorial pole at the annular pad and, afterwards, elongate and differentiate to nucleated fiber cells and secondary fiber cells.

dynamic tailoring of the glycan sequence and of the local density of cognate epitopes opens the way to modulate their properties as (ga)lectin ligands. Most notably, occupancy of the 6'-OH group of the galactose unit (by sialylation) precludes an interaction with a galectin so that α2,6-sialylation is like a molecular switch-off mechanism. In principle, to reach the aim of building a versatile recognition system operative at many sites, presentation of various types of ligands and intimately regulated expression of several members of the galectin family compose the contents of a toolbox that is ideal to generate a large quantity of permutations

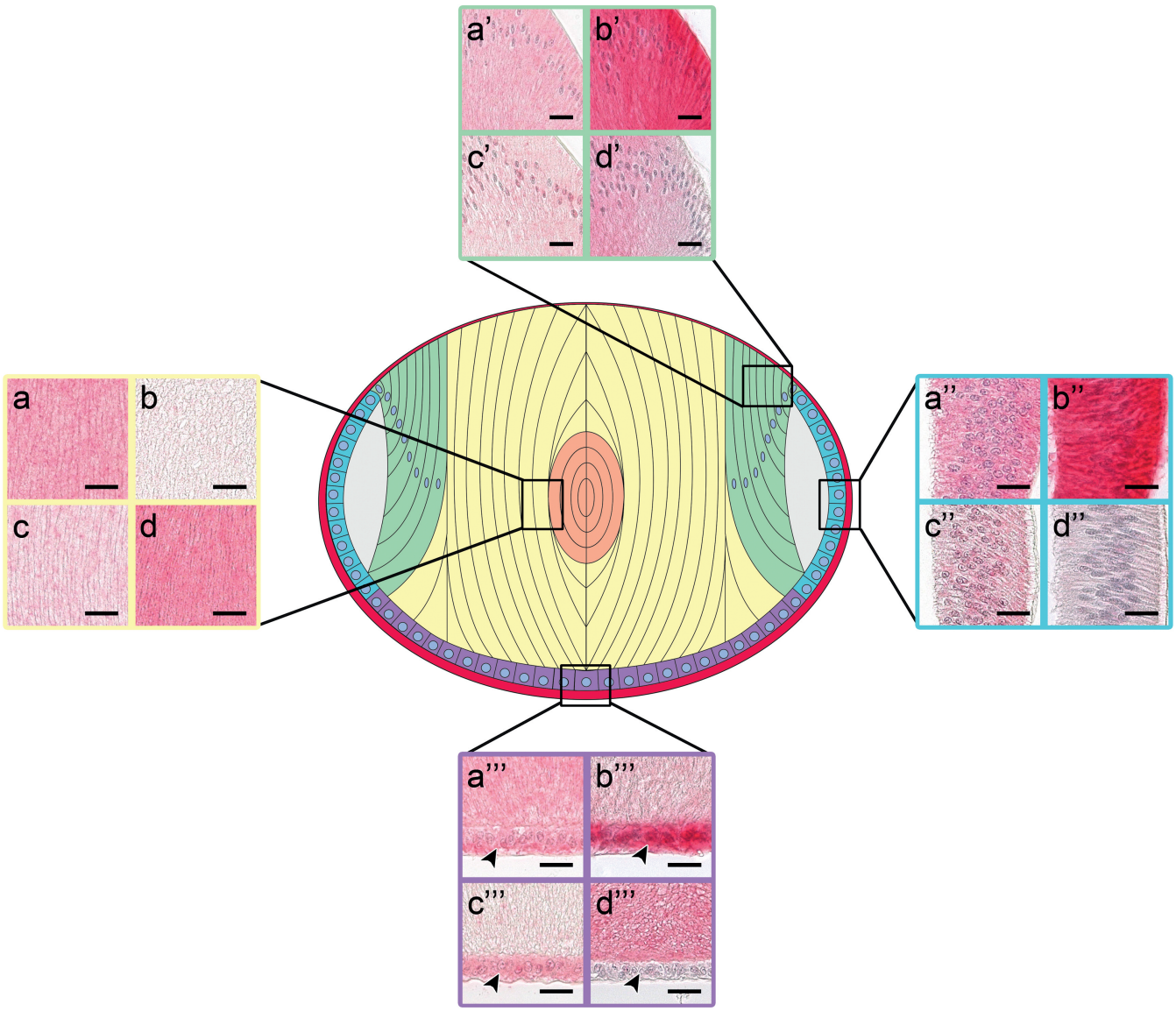


Fig. 6. Schematic representation of an eye lens of a chicken embryo at HH stage 36 together with photomicrographs that illustrate distinct staining patterns for localization of CG-1A (**a-a'''**), CG-3 (**b-b'''**), CG-8 (**c-c'''**) and C-GRIFIN (**d-d'''**) in different regions of the lens (secondary (yellow)/primary (orange) lens fibers (**a-d**); transitional zone (green, **a'-d'**); annular pad (blue, **a''-d''**) and central anterior epithelium (magenta, arrowheads in **a'''-d'''**)). Processing of sections with the specific anti-CG-1A IgG fraction led to moderate homogeneous staining in secondary/primary fiber cells (**a**), nucleated fiber cells at the transitional zone (**a'**), equatorial epithelial cells of the annular pad (**a''**) and central anterior epithelial cells (**a'''**). In contrast, strong signals for presence of CG-3 were detected in nucleated fiber cells (**b'**) and anterior/equatorial epithelial cells (**b'', b'''**), whereas secondary and primary fiber cells were only weakly stained (**b**). Low signal intensity for CG-8 presence was observed in fiber cells (**c, c'**) and, comparatively stronger, in epithelial cells at the annular pad (**c''**) and cells of the anterior central epithelium (**c'''**). Presence of C-GRIFIN was restricted to fiber cells, with strong staining intensity in primary and secondary fiber cells (**d**) and moderate intensity in nucleated fiber cells at the transitional zone (**d'**). Antibody concentrations: 1 μg/mL for anti-CG-1A and anti-CG-3; 4 μg/mL for anti-CG-8 and anti-C-GRIFIN (for details on CG expression in the eye lens during the course of development, please see García Caballero et al., 2020). Scale bars: 20 μm.

en route to multifunctionality.

When examining Fig. 7 accordingly, the number of the types of β-galactosides (and also two glycans, in which N-acetylgalactosamine (GalNAc) takes the place of galactose) used as headgroups and known to be ligands for members of the galectin family is indeed high. A large panel of methods has been helpful to collect information on affinity, selectivity and specificity in glycan-galectin recognition (for an overview of the techniques, please see Table 1 in Solís et al., 2015; for selected illustrations of experimental data obtained in assays with human Gal-1, please see Fig. 4 in Gabius et al., 2011; for an example of testing galectin specificity with synthetic glycocompounds histochemically, please see Roy et al., 2017; for an overview on binding profiles for glycans measured by frontal affinity chromatography,

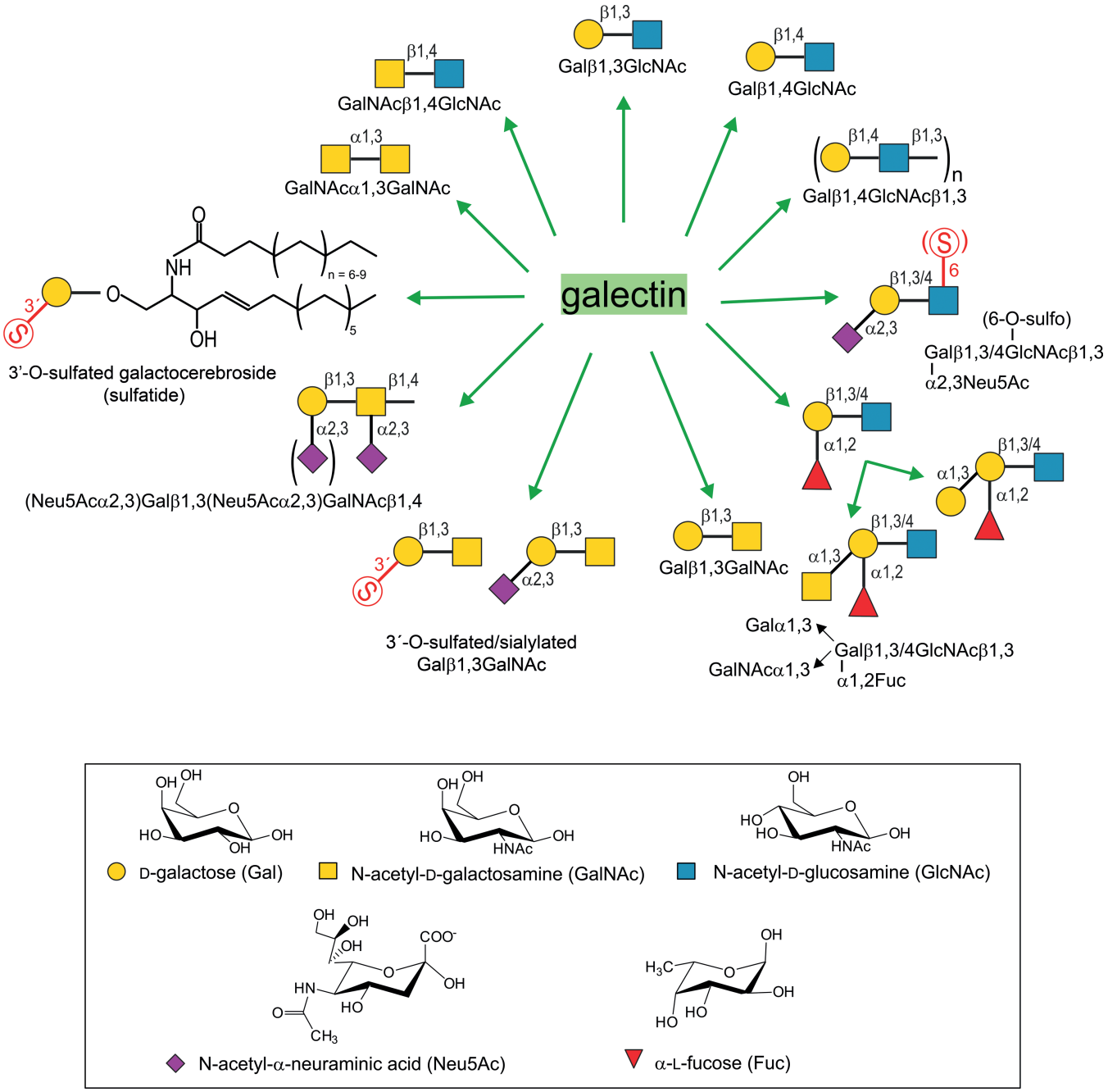


Fig. 7. Illustration of the structures of di- to tetrasaccharides and the headgroup of sulfatide that are known as galectin ligands (symbols used for carbohydrates are explained in the bottom part).

please see Iwaki and Hirabayashi, 2018; for a survey on the architecture of the contact region between glycans and mammalian galectins beyond Gal-1, please see Romero and Gabius, 2019). Notably, structural features such as presentation of N-acetyllactosamine in tandem repeats (Galβ1,4GlcNAcβ1,3; LacNAc) in especially β1,6-linked chains of complex-type N-glycans (added by GnT-V) or core 2/4 mucin-type O-glycans (added by C2GnT), introduction of a sialic acid moiety or a sulfate group at the 3'-position of galactose or substitution of the LacNAc core to build histo-blood ABH group determinants will shift affinity values between galectins. As a consequence, often seemingly subtle structural alterations among β-galactosides endow cells with a graded responsiveness to individual galectins. Toward the same aim, the topology of glycan presentation can be altered.

A potent means to increase the affinity of a galectin to a glycan ligand is to arrange galactose-containing headgroups in clusters, either by local vicinity of headgroups in branched N- or O-glycans, as determined for a glycoprotein (asialofetuin) with three triantennary N-glycans (Dam et al., 2005), or by microdomains, whose high-affinity binding capacity to Gal-1 and -3 is impaired by disrupting their integrity by extracting cholesterol (Kopitz et al., 2010). Interestingly, loading of the binding sites on a glycoprotein with galectin proceeds in a gradient of decreasing affinity constants, letting occupancy of the first site(s) attain (very) high fractional affinity (Dam et al., 2005). Local clustering of sites suited for contact defined by the glycans' sequence and also their conformer status (the third dimension of the sugar code following structural aspects of linear and branched glycan chains as first and second dimensions; von der Lieth et al., 1998; for detailed information on conformers of the pentasaccharide of ganglioside GM1 and their (differential) selection by lectins, please see Ledeen et al., 2018) can therefore guide a galectin to its high-preference target in the specific context, and this can not only happen on cell surfaces and in the ECM but also in the cell.

In addition to glycoconjugates at extracellular sites, pairing of a galectin with glycans is also possible intracellularly. What has puzzled researchers in the field for decades, i.e. intracellular galectin presence without an obvious glycan to bind to, is now making sense. Currently attracting enormous attention, damage to membranes of endocytic vesicles or phagosomes can make glycans accessible to galectins such as Gal-3 and -8 in the cytoplasm. Their association to these docking sites can then initiate the autophagy program in response to harm done to endomembranes by serving as a core for the formation of a complex together with tripartite motif 16 (TRIM16), an E3 ubiquitin ligase and link to autophagy mediators such as ULK1 or Beclin-1 (for a review on intracellular galectins as effectors to start autophagy programs, please see Hong et al., 2018; for more details on peptides as docking site for galectins, please see the next section). In these cases, the glycan that is now accessible signals danger, its binding by a galectin is followed by its interaction with a protein of the autophagy response: intracellular galectins sense danger and call for help. This apparent bifunctionality of first binding a glycan, thereafter a protein is – *mutatis mutandis* – evocative of the interplay between two sites in a slime mold lectin (discoidin I) to exert its crucial role in the ordered cell migration upon starvation (Gabius et al., 1985). In our context, this intracellular galectin activity embodies one route to multifunctionality by involving more than one contact region within the CRD, a clear case of moonlighting (for more details on peptides as ligands, please see the next section).

As noted at the end of the previous section, the application of (plant/fungal) lectin histochemistry has taught us that glycan profiles are subject to change in the course of cellular processes such as differentiation or acquisition of the malignant phenotype. Plant, fungal and bacterial lectins have all been used as tools to detect respective alterations, and such data make us wonder why these differences actually occur. The work on the endogenous lectins that likewise have become tools in cyto- and histochemistry (for an example on Gal-1, please see Gabius et al., 1991) has uncovered an important clue. All in all, shifts in the composition of the cellular glycome alter its capacity to interact with tissue lectins. Differentiation/disease-associated epitopes could become functional markers, if a tissue lectin could be found that 'reads' such a signal and then 'translates' its meaning into a (patho)physiological consequence. The case of the three C-type lectins known as selectins and the co-expression of counterreceptors for them in inflammation and cancer progression has already furnished striking proof for intimately regulated functional pairing within the concept of the sugar code (McEver, 1997; Ley, 2003; Borsig, 2018; Ivetic, 2018). Indeed, several molecular mechanisms have already been discovered that verify this fundamental hypothesis for galectins, as now given below.

An often encountered modality to alter the glycophenotype is removing or adding a sialic acid residue from glycan chains. Actually, this type of glycan remodeling had been detected in the course of neuroblastoma cell differentiation or effector T cell activation, and it had been traced back to the activation of a cell surface ganglioside neuraminidase (Kopitz et al., 1994, 1997; Wang et al., 2009). This enzyme converts the hexasaccharide of ganglioside GD1a by a desialylation step to the pentasaccharide of ganglioside GM1, a counterreceptor for Gal-1 and therefore a signal for growth control (Kopitz et al., 1998, 2001; for reviews, please see Ledeen et al., 2012, 2018). Intriguingly, enzymatic downsizing transforms a masked glycan into a compact molecular 'message' that is 'read' by a galectin resulting in reduced cell proliferation.

The same aim, that is making such a signal accessible, is reached by turning down the extent of sialylation, often seen to be high on tumor cell surfaces,

on the level of the transfer of sialic acid from the donor (CMP-sialic acid) to the acceptor (glycan chain) by a sialyltransferase. α2,6-Sialylation of N-glycans in (pancreas) carcinoma cells can be interpreted as a means to make binding and therefore growth regulation by a galectin impossible. If this is the case, then decreasing the level of presence of α2,6-linked sialic acids could make tumor cells susceptible to growth control by galectin(s). Indeed, the tumor suppressor $p16^{INK4a}$ was shown to instruct pancreatic carcinoma (Capan-1) cells to revert from malignant growth to being responsive to Gal-1 to undergo anoikis this way. The tumor suppressor's negative effect on sialic acid synthesis (by downregulation of mRNA and/or protein levels of two enzymes of this pathway, i.e. GNE and NANS) and the resulting decrease in degree of α2,6-sialylation of N-glycans are the reasons why surface glycoproteins of $p16^{INK4a}$-positive tumor cells bind Gal-1 better, as shown by cytofluorometry: anoikis is then induced by the pairing of Gal-1 with the fibronectin receptor ($\alpha_5\beta_1$-integrin), which in a cascade first causes activation of focal adhesion kinase and in the next step of caspase-8 (André et al., 2007; Amano et al., 2012). Strikingly and making sense pathophysiologically, the extents of presence of Gal-1 (and also of the $\alpha_5\beta_1$-integrin) on the cell surface are increased at the same time in a concerted manner. Hereby, compelling evidence is provided for a co-regulation of glycan and protein (to generate the productive glycoprotein counterreceptor) as well as of the galectin: its binding via the cognate glycan cross-links the effector glycoprotein to set the stage for downstream signaling. Distinct glycans, by virtue of their presentation by a particular protein and their interaction with a tissue lectin, hence qualify as functional biomarkers. In aggregate, the dynamic remodeling generates both a phenotypic characteristic, in the case of α2,6-sialylation of N-glycans cyto- and histochemically readily detectable by a fungal lectin (i.e. PSL, from *Polyporus squamosus*; Mo et al., 2000; Toma et al., 2001) or by human siglec-2 (CD22) (Lohr et al., 2010), and a sugar-based signal 'read' and 'interpreted' by a galectin.

In addition to the dynamic remodeling via a glycosidase or by substrate availability, glycosyltransferases are already known whose expression affects tumor cell properties. Their activities implement new characteristics in the glycome, with relevance for galectin binding. With focus on O-glycosylation, introduction of a β1,6-branch in mucin-type O-glycans (by C2GnT) and ensuing polyLacNAc addition (by alternating action of a β4GalT and a β3GnT) were associated with induction of Gal-1-dependent cell death in T lymphoma cells (Galvan et al., 2000; Cabrera et al., 2006). An efficient attack on bladder cancer cells by cytotoxic T lymphocytes via HLA class I recognition occurred by prolonging its surface presentation in a lattice that Gal-3 holds together (Sutoh Yoneyama et al., 2017), whereas natural killer (NK) cell silencing happened, if Gal-3 bound to the polyLacNAc extension of O-glycans of MHC class I-related chain A interferes with tumor cell-NK cell (here the activating receptor NKG2D) recognition (Tsuboi et al., 2011).

Intriguingly (but not unexpected), keeping O-glycans small by making branching impossible can have remarkable consequences. Introducing a sialic acid moiety via an α2,6-bond into mucin-type core 1 O-glycans at the early stage of O-glycosylation, that is into the Ser/Thr-linked GalNAc moiety (by ST6GalNAc-II), is referred to as a suppressor mechanism for breast cancer metastasis. The presence of this sialic acid residue precludes enzymatic extension of the so-called T_n antigen (T_n="T antigen nouvelle", αGalNAc, CD175) to the Thomsen-Friedenreich disaccharide (TF antigen, Galβ1,3GalNAcα, CD176; for reviews on T_n/T antigens, please see Berger, 1999; Ju et al., 2011; Gabius et al., 2015), a galectin ligand shown in Fig. 7. Therefore, Gal-3 binding is prevented that otherwise would both elevate tumor cell retention in lung and cell interactions establishing emboli (Murugaesu et al., 2014). Also, α2,3-sialylation of the TF epitope on tumor cells will then be precluded. This process would otherwise produce a ligand for siglec-9 expressed on myeloid cells, this glycan-lectin interplay also favoring tumor progression (Beatson et al., 2016; Fraschilla and Pillai, 2017).

Toward the same end, the loss of expression of the specific protein to present the cognate glycan and to convey its message can occur. Reflecting clinical significance, this is the case for CD7: accumulation of CD7-negative T lymphoma cells is often seen during disease progression in patients who suffer from Sézary syndrome. This phenomenon is likely, at least in part, due to these cells' resistance to Gal-1-induced cell death, because pairing of Gal-1 with CD7 is essential to trigger apoptosis in these tumor cells (Rappl et al., 2002). Overall, these examples on affecting glycan and glycoconjugate display illustrate the emerging intimate interplay between cellular glycosylation and galectins.

To test this relationship quantitatively in a bottom-up manner, a box of bricks for building custom-made model test systems would be ideal. Design and application of nanoparticles (obtained by self-assembly of amphiphilic Janus glycodendrimers) with chemically programmable surface glycan profile have already proven very helpful for this purpose (Percec et al., 2013). The possibility to vary glycan density and structural complexity systematically generates versatile tools to answer questions for example on activity differences among galectins (Zhang et al., 2014; Xiao et al., 2018). In addition, as already indicated above, natural galectin variants are based on either alternative splicing or single nucleotide polymorphism, as is the case for Gal-8, a bridging factor between myeloma and vascular endothelial cells (Friedel et al., 2016), and respective protein pairs have already been tested with glycodendrimersomes to reveal first evidence for functional differences by linker length variation (33 or

74 amino acids) and occurrence of the F-to-Y transition at position 19 (Ruiz et al., 2014; Zhang et al., 2015).

Of conspicuous significance for the (patho)-physiological multifunctionality of galectins, glycans as docking sites for galectins can be attached to a multitude of natural scaffolds. In other words, the glycosylation machinery can add the label for galectin binding to many proteins: when this happens to cell-type-specific proteins, then post-binding effects are locally restricted, and the elicited signaling can in principle take a host of directions depending on the nature of the target glycoconjugate. The expression of galectins at various sites, noted above, is then instrumental to make context-specific pairings possible. Fittingly, galectins (and other types of tissue lectins) appear to be selective among the cellular glycoconjugates (Gabius et al., 2016). In other words, the nature of the target glycoconjugate(s) for a galectin can vary with the cell type (for a compilation of documented glycoconjugate counterreceptors for Gal-1 and -3, please see Table 2). Hereby, phrased as a take-home message, the same galectin is able to interact with components in diverse pathways to exert its pleiotropic effects noted above, for example an integrin (on carcinoma cells; Sanchez-Ruderisch et al., 2011) or the mentioned glycoprotein CD7 (on activated or leukemic T cells). In addition, as already indicated above in the context of growth regulation via glycan-inhibitable or -insensitive cell binding of Gal-1, the Gal-3 and -8-dependent initiation of autophagy and crystallin association to eye-lens-specific GRIFIN, galectins do bind motifs other than the β-galactosides depicted in Fig. 7.

Toward multifunctionality: peptides as ligands

The interaction of galectins with peptides is not confined to very few cases. Table 2 presents known peptide-based ligands for Gal-1 and -3 along with cognate glycoconjugates. The contact between the two partners of such a recognition process can involve different regions within the β-sandwich fold of a galectin, even a part of the section that accommodates the canonical glycan ligand lactose. In that case, the presence of the NWGR motif in Gal-3 (with the Trp engaged in the C-H/π-based molecular rendezvous with galactose and the following Arg residue, both belong to the signature sequence; please see Fig. 1), which is known to be a part of the BH1 domain of the Bcl-2 family of proteins, the lactose-inhibitable association of Gal-3 with Bcl-2 and the loss of this galectin's anti-apoptotic activity by the G-to-A mutation verify the assumption of a functional significance of this part of the sequence signature for a peptide-based Gal-3–Bcl-2 interaction in the cytoplasm (Yang et al., 1996; Akahani et al., 1997).

When in the nucleus, Gal-3 is able to upregulate activity of distinct promoters, for example of the cyclin D1 promoter (Lin et al., 2002). Physical interaction with a transcription factor, i.e. the thyroid-specific transcription factor (TTF)-1, or a role in pre-mRNA splicing add to the already documented nuclear activities (Park et al., 2001; Paron et al., 2003; Nakahara and Raz, 2007). The interaction of Gal-1 with the transcription factor TFII-I, a general component of spliceosomes, did yet not require an essential amino acid for lactose binding, i.e. N46, because the N46D mutant was as active as the wild-type protein (Voss et al., 2008). Proceeding from mutagenesis to physical mapping, interaction analysis by (^{1}H-^{15}N HSQC) NMR spectroscopy using isotopically labeled protein is a means to identify the actual contact sites. By performing

Table 2. Survey of cellular glycoconjugates and proteins that have been identified as counterreceptors for human galectin-1 (Gal-1) and galectin-3 (Gal-3).

Type of ligand	Gal-1	Gal-3
Glycoconjugate	ovarian carcinoma antigen CA125, CD2, CD3, CD4, CD6, CD7, CD43, CD45, CD69, CD95(Fas), CD146 (MCAM, MUC18), CD166 (ALCAM), carcinoembryonic antigen (CEA), fibronectin (tissue), gastrointestinal mucin, hsp90-like glycoprotein, β_1-integrin (CD29), $\alpha_1/\alpha_4/\alpha_5/\alpha_7\beta_1$-, $\alpha_{IIb}\beta_3$- and $\alpha_4\beta_7$-integrins, cell adhesion molecule L1, keratan sulfate, laminin, lamp-1, Mac-2-binding protein, nephrin, neuropilin-1, receptor protein-tyrosine phosphatase (RPTPβ), thrombospondin, Thy-1, tissue plasminogen activator, von Willebrand factor, chondroitin sulfate proteoglycan, distinct neutral glycolipids, ganglioside GM1	CD6, CD7, CD11b of CD11b/CD18 (Mac-1 antigen, CR3), CD13 (aminopeptidase N), CD32, CD43, CD44, CD45, CD66a,b, CD71, CD95, CD98, CD146 (MCAM, MUC18), CD147, CD166 (ALCAM), CEA, colon cancer mucin, corneal mucin (MUC16), pancreas cancer mucin-4 and MUC1-D (N-glycan at Asn36), cubilin, C4.4A (member of Ly6 family), mDectin-1/2, desmoglein-2, epidermal growth factor receptor, glycoform of IgE, haptoglobin β-subunit (after desialylation), hensin (DMBT-1), insulin receptor, insulin-like growth factor-1 receptor, β_1-integrin (CD29), $\alpha_4/\alpha_5/\beta_1$- and $\alpha_v\beta_3$-integrins, interferon-γ, keratan sulfate, LI-cadherin, laminin, lamp-1/-2, lubricin, Mac-2-binding protein, Mac-3, MAG, MP20 (tetraspanin), Na^+/K^+-ATPase, NG2 proteoglycan, NKp30, TCR complex, tenascin, tissue plasminogen activator, SIGN-R1, Toll-like receptor-4, transforming growth factor-β receptor, vascular cell adhesion molecule-1, vascular endothelial growth factor receptor 2, von Willebrand factor, ganglioside GM1
Protein	B lymphocyte adaptor molecule of 32 kDa (Bam32), CaV1.2 L-type calcium channel (α_1-subunit), FOXP3, Gemin4, oncogenic H-Ras/RBD of C-Raf , OCA-B, pre-B cell receptor (human, not murine system), transcription factor TFII-I	AGE products, Alix/AIP-1, ATP synthase. b-subunit, axin, Bax, Bcl-2, β-catenin, CXCL12, Cys/His-rich protein, Gemin4, glycogen synthase kinase-3β, hnRNP A2B1, hnRNP Q, mSufu, Mer receptor tyrosine kinase, myosin-2A, NLRP3 (NOD-like receptor family, pyrin domain containing 3, cryoporin), non-receptor tyrosine kinases c-Abl and Arg, nucleoporin Nup98, nucling, oncogenic K-Ras, OCA-B, pCIP, PIAS1, synexin (annexin VII), Tsg101, thyroid-specific transcription factor (TTF)-1, transcription factor TFII-I, tripartite motif protein (TRIM) 16 (and also 5α, 6, 17, 20, 22, 23 and 49)

this type of experiment, the binding of the pre-B cell receptor to Gal-1 was revealed to engage entirely independent sites of interaction for this protein ligand and for lactose (Elantak et al., 2012), terminologically entirely correct moonlighting by the galectins ensured. Remarkably, Gal-1 is also a binding partner of OCA-B, known as a B cell-specific transcriptional coactivator involved in immunoglobulin gene expression and a signaling molecule (Yu et al., 2006). In addition to peptides, binding to the farnesyl chain (of oncogenic H-Ras) in the case of Gal-1 (Rotblat et al., 2004; Yu et al., 2015) and to nucleic acids in the case of Gal-3 (Wang et al., 1995) is documented. In this special instance, the strongly basic isoelectric point of Gal-3 (as also exhibited by Gal-8) may play a role.

In summary, the CRD of galectins has more to offer as a platform for interactions than the canonical affinity to β-galactosides. Cognate peptide motifs add proteins to the growing list of counterreceptors of galectins. Each galectin – due to the sequence divergence – has its own specificity spectrum (and even very closely related galectins such as human Gal-1 and -2 that share 43% sequence identity differ in profile of binding partners and of triggered effector mechanisms, here stimulation of non-uniform sets of caspases in activated T cells; Sturm et al., 2004), as is the case for the expression and localization profiles. To reach multifunctionality, the already discussed factors would already suffice but – last but not least – there is a further feature of enormous functional significance, i.e. the architecture of galectins.

Toward multifunctionality: galectin architecture

The experimental read-out in the popular haemagglutination assay is based on a distinct aspect of lectin architecture, i.e. the ability to bridge ligands on opposing cell surfaces. In functional terms, cross-linking by galectins will also be the basis to bring counterreceptors together in lattices on membrane surfaces. This arrangement slows down endocytosis, which prolongs residence time of the cognate glycoproteins on the cell surface, as has been mentioned above as a consequence of HLA class I–Gal-3 recognition on bladder tumor cells (Sutoh Yoneyama et al., 2017). Along this line and of conceivable clinical perspective for type 2 diabetes, a probable link between diet and insulin production by glycosylation of a distinct surface glycoprotein has been reported in an animal model: rate of endocytosis of the pancreatic β cell glucose (Glc) transporter 2 (causing its removal from the plasma membrane) was enhanced by attenuation of N-glycan branching (by GnT-IVa); less branching is assumed to account for a decrease in extent of aggregate formation of this glycoprotein with galectins such as Gal-9, probably crucial for shortening the Glc transporter's surface residency (Ohtsubo et al., 2005). Building of high-order galectin-glycoconjugate complexes also is the decisive step toward post-binding signaling, as for instance triggered *en route* to a mitogenic stimulation, and "the same may also be true for the induction of other biological effects in cells by lectins" (Sharon, 1994). Since the galectin CRD contains a single contact site for β-galactosides, cross-linking can only be achieved by somehow forming CRD clusters, and here galectin architecture comes into play.

Actually, the CRD display in vertebrate galectins looks simple but is strategically highly sophisticated, a graphical overview given in Fig. 8. As shown, three types are established: a non-covalent association of the same type of CRD (proto type), a linker-based (thus

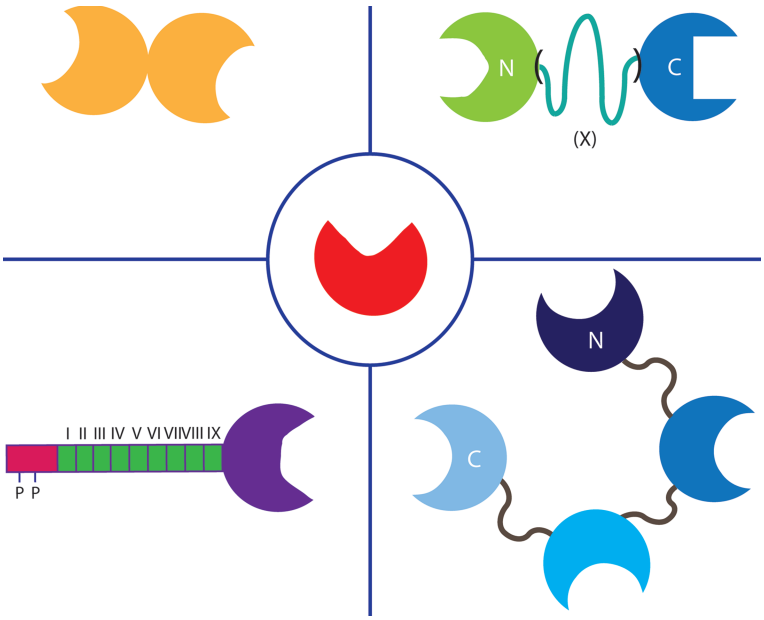
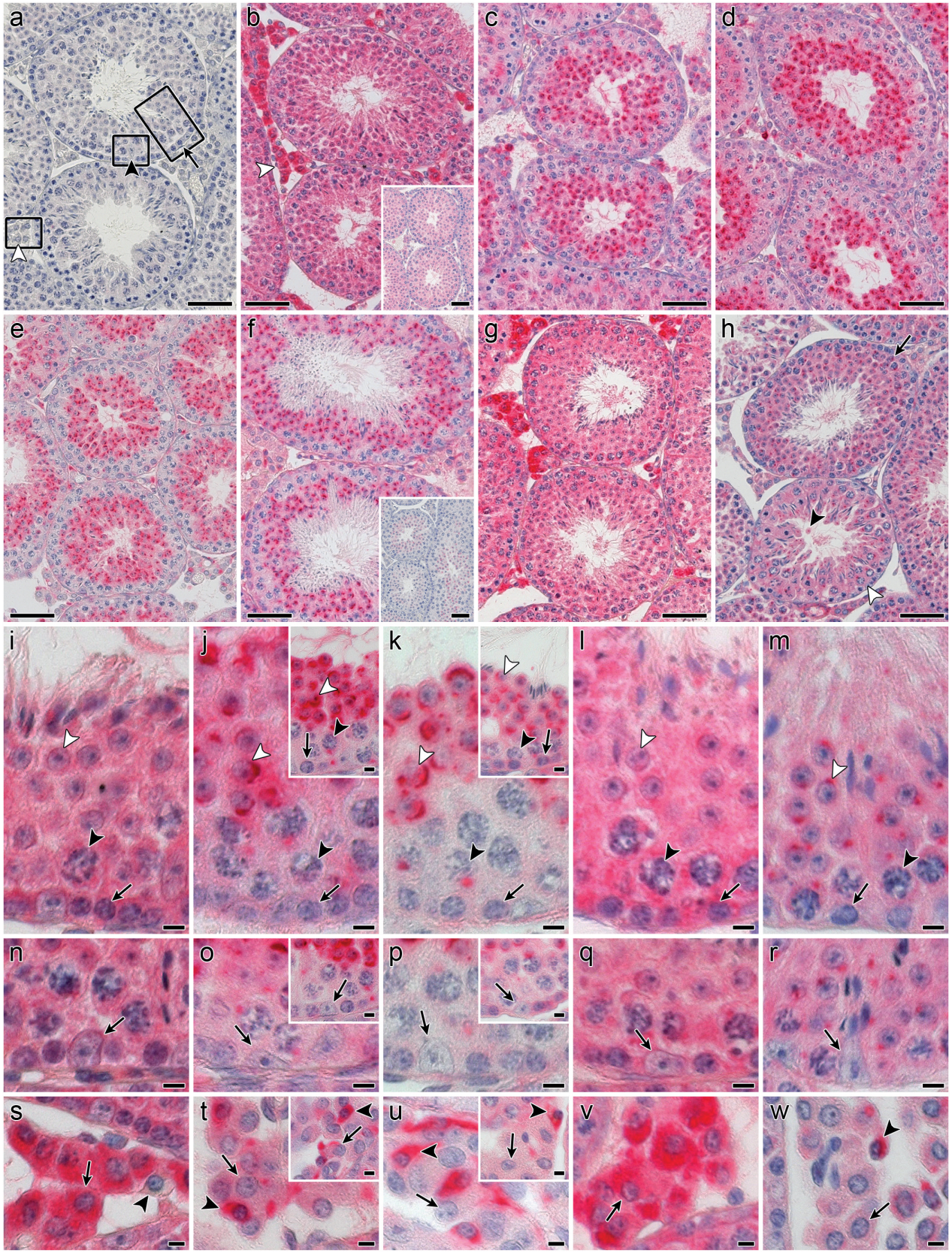


Fig. 8. Illustration of types of architecture of (in)vertebrate galectins that originate from the ancestral carbohydrate recognition domain (CRD; center), as listed in Fig. 2 (choice of color reflects homology up to identity of CRDs in the proto-type (homodimeric) proteins). Non-covalently associated homodimers are called 'proto-type galectins', linker-connected heterodimers (and hetero-oligomers) are 'tandem-repeat-type galectins' and a C-terminal CRD connected to an N-terminal section with phosphorylation sites and collagen-like repeats is 'chimera-type galectin' (Gal-3 in vertebrates); for details on chicken galectins, please see Fig. 4 in Kaltner and Gabius, 2012.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covalent) association of two different types of CRD (tandem-repeat type) and a modular design, in which the CRD is connected to an N-terminal segment with two sites of serine phosphorylation (relevant for nuclear transport) and a section established by non-triple-helical collagen-like repeats (relevant for aggregate formation) to build a chimera-type galectin, in Gal-3 (Fig. 8). These classifying terms have already been entered into Fig. 2, which in boxes tabulates the presence of representatives of each group and the variability in numbers among selected animals. What's more, with respect to the types of galectin design, exchange of CRDs is possible among individual homodimeric galectin proteins. This was first shown for rat GRIFIN (Ogden et al., 1998), and CRDs can even switch partners to create heterodimers (Miller et al., 2018).

The given panel of three types of galectin design means that diverse combinations of counterreceptors can be included into lattices, and, beyond this, that lattices of different spatial organization arise by either involving a bi- or a multivalent galectin. Actually, Gal-3 is unique among galectins by being able to turn its monomer into aggregates using contacts between the N-terminal tail and/or between CRDs or both (for a survey on the structural basis of Gal-3 aggregation, please see Flores-Ibarra et al., 2018; for experimental determination of the tail's critical length for aggregation, please see Kopitz et al., 2014). This possibility for generating galectin-glycoconjugate complexes of non-identical topological order had first been discovered for the proto-type Gal-1 vs the chimera-type Gal-3 (Ahmad et al., 2004). The differences in protein architecture combined with sharing affinity to the same counterreceptor can then be the basis for functional antagonism. Indeed, blocking Gal-1 activity by Gal-3 was found for these two galectins in tumor cell growth regulation (Kopitz et al., 2001; Sanchez-Ruderisch et al., 2010) and in nanoparticle aggregation (Ludwig et al., 2019a). When shared bivalency is combined with disparity in target selection, in contrast, this scenario can lead to different post-binding effects, tandem-repeat-type Gal-4 being unable to inhibit neuroblastoma cell growth *in vitro* that homodimeric Gal-1, -2 and -7 all reduce (Kopitz et al., 2003, 2012). In contrast, its special affinity to sulfatides, especially with long-chain (C24) fatty acids, predestines it as cargo transporter in apical or axonal delivery of glycoproteins (Delacour et al., 2005; Stechly et al., 2009; Velasco et al., 2013). Since vertebrates have genes for members of each of the three design groups (Fig. 2), the arising potential for (complex) functional interactions *in situ* has intriguing implications for the study of galectin expression. Obviously, a comprehensive network analysis is essential to delineate all possibilities for such an interplay by co-expression.

Equally relevant as antagonism is, cooperation toward the same read-out, i.e. manifestation of pro-degradative/inflammatory effects via activation of the NF-κB pathway in osteoarthritis, has been documented. Testing mixtures of Gal-1, -3 and -8 in line with their immunohistochemically detected co-upregulation in osteoarthritis pathogenesis revealed this type of functional cooperation (Toegel et al., 2014; Weinmann et

Fig. 9. Illustration of galectin histochemical staining profiles in cross sections through fixed murine testis. Photomicrographs document overviews (**a-h**), and regions at increased magnification (**i-w**) are presented for distinct cell types (i.e. cells of the spermatogenic cycle (**i-m**): the respective part of the tubule analyzed is indicated as a boxed area marked with an arrow in panel **a**; Sertoli cells (**n-r**): the part of the tubule analyzed is designated by the boxed area marked with a filled arrowhead in panel **a**; Leydig cells (**s-w**): the part of the intertubular interstitium analyzed is highlighted by the boxed area marked with an open arrowhead in panel **a**. **a.** Negative control by omission of the incubation step with first-step reagent (labeled galectin) to exclude lectin-independent signal generation. **b.** Strong binding of wild-type Gal-1 in the cytoplasm of cells of the seminiferous epithelium and comparatively stronger positivity in Leydig cells (white arrowhead). **Inset to b** exemplarily shows extent of reduction of galectin binding by incubation of labeled probe in the presence of cognate sugar (lactose; 200 mM). **c-f.** The linker-connected variant proteins stained round spermatids, particularly strong in the Golgi and the acrosomal cap region. In the cytoplasm of spermatogenic cells, the degree of intensity was stronger for $(Gal\text{-}1)_2$-GG **(c)** and for $(Gal\text{-}1)_2$-8S (**d**) than for the respective tetramers, i.e. $(Gal\text{-}1)_4$-GG (**e**) and $(Gal\text{-}1)_4$-8S (**f**). Presence of 10 mM lactose nearly completely inhibited binding of $(Gal\text{-}1)_4$-8S (**inset to f**). **g.** Processing with labeled Gal-3-like Gal-1, i.e. Gal-3NT/1, generated a staining profile and degree of intensity comparable to those obtained with wild-type Gal-1 (**b**), whereas binding sites for wild-type Gal-3 were detected at moderate level of staining in the cytoplasm of cells of the seminiferous epithelium (**h**). The Golgi region of spermatogenic cells in stage VII (arrow)/XII (open arrowhead) of the spermatogenic cycle and residual bodies (filled arrowhead) within the epithelium of a stage XII tubule section showed prominent dot-like staining (**h**). Enlarged views of the main cell types (please see corresponding boxed areas in **a**) document nearly identical staining profiles for labeled wild-type Gal-1 (**i, n**) and Gal-3NT/1 (**l, q**) with a strong and rather homogeneous cytoplasmic positivity of cells of the spermatogenic cycle (round spermatid, open arrowhead; spermatocyte, filled arrowhead; spermatogonia, arrow; **i, l**) and of Sertoli cells (arrows; **n, q**). Leydig cells (arrows, **s, v**) were strongly positive, whereas interstitial macrophage-like cells (filled arrowhead, **s, v**) were negative. **j, k.** Processing of sections with labeled homodi- and tetrameric variant protein, i.e. $(Gal\text{-}1)_2$-GG **(j)**, $(Gal\text{-}1)_2$-8S (**inset to j**), $(Gal\text{-}1)_4$-GG (**k**) and $(Gal\text{-}1)_2$-8S (**inset to k**), led to very strong staining intensity in acrosomal caps, cytoplasm and Golgi region of round spermatids, and, occasionally, in the Golgi region of spermatocytes (filled arrowheads). Cytoplasm of spermatocytes (filled arrowheads), spermatogonia (arrows) (**j, inset to j**), Sertoli cells (arrows, **o, inset to o**) and Leydig cells (arrows, **t, inset to t**) presented weak staining intensity after testing $(Gal\text{-}1)_2$-GG and $(Gal\text{-}1)_2$-8S. Reduced level in staining intensity was observed in these cell types when applying $(Gal\text{-}1)_4$-GG (**k, p and u**) and $(Gal\text{-}1)_4$-8S (**insets to k, p and u**). Notably, macrophage-like cells in the intertubular interstitium (filled arrowheads in **t, inset to t; u, inset to u**) were stained by the labeled variants with strong intensity. Binding of wild-type Gal-3 was detected in the cytoplasm of the cells of the spermatogenic epithelium (spermatids, open arrowhead; spermatocytes, filled arrowhead; spermatogonia, arrow in **m**, Sertoli cells, arrow in **r**) at a moderate and in the cytoplasm of Leydig cells (arrow, **w**) at a low level. Golgi regions of round spermatids (open arrowhead, **m**) and of macrophage-like cells in the intertubular interstitium (filled arrowhead, **w**) were strongly positive for Gal-3 binding. The following concentrations were applied: Gal-1: 0.25 μg/mL; $(Gal\text{-}1)_2$-GG, $(Gal\text{-}1)_2$-8S, $(Gal\ 1)_4$ GG, $(Gal\text{-}1)_4$-8S, Gal-3NT/1: 0.5 μg/mL; Gal-3: 8.0 μg/mL. Scale bars: a-h, 50 μm; n-w, 5 μm; for details on technical protocol, please see Kutzner et al., 2019.

al., 2018). This proof-of-principle evidence for functional additivity/synergy and antagonism between galectins highlights the interest to routinely combine mapping galectin presence with functional assays using mixtures. Besides its comparatively small group size, as indicated above, the representation of all three types of design for the CGs is another reason to study their expression in detail as a model with relevance for mammalian galectins.

When looking at Figs. 2, 8 the fundamental question arises as to why vertebrates express galectins of these distinct types of architecture and not for example the covalently linked homotetramers of Mollusca. As a step toward finding an answer to this problem, oligomers with the CRD of human Gal-1 as building block were created. By respective protein engineering on the level of the CRDs to alter galectin architecture purposefully, termed lectinology 4.0 (Ludwig et al., 2019b), variants were obtained and tested functionally along with the wild-type proteins. This approach revealed effector activity of the homotetramer with the CRD of human Gal-1 already at low levels of counterreceptor (ganglioside GM1) presence, which will likely compromise the physiological functionality of the mentioned GD1a-to-GM1 conversion in cell growth regulation (Kopitz et al., 2017). In contrast, the very potent aggregation of microbial pathogens by the natural homotetramer can contribute to protect the oyster in an environment replete of such challenges (Tasumi and Vasta, 2007). Elucidating functions of such other types of design can thus inspire innovative ideas to design and to test variants based on a CRD of a human galectin for medical applicability.

Traveling along this route, any CRD can become a part of an established architecture, a productive approach to define the significance of the architecture for function, as explained here for Gal-3: when the chimera-type protein acquired bivalency like Gal-1, the resulting homodimer of the Gal-3 CRD was no longer an antagonist of the growth-inhibitory Gal-1. Instead, it was as active as negative regulator as the Gal-1 homodimer (Ludwig et al., 2019a). Flanking such proliferation assays experimentally, the effect of the redesign of a natural CRD arrangement can also be tested by lectin histochemistry. This study line has been initiated for linker-connected Gal-1 homodi- and tetramers and a Gal-3-like Gal-1 protein (Kutzner et al., 2019). Examples for the impact of altering the protein design from the wild-type version to variants of human Gal-1 on the characteristics of cell staining are given in Fig. 9 and Fig. 10, together with a summary of the staining profiles in murine testicular tissue, which is presented in Table 3. Obviously, already the introduction of a short linker (Gly-Gly) into the Gal-1 homodimer leads to changes relative to non-covalently associated wild-type Gal-1 that are clearly seen for Leydig and macrophage-like cells (Fig. 9a-f,i-k,n-p,s-u). Whether one can attribute a shuttling of the Gal-1 CRD into a new structural context to deviations in staining relative to the respective wild-type galectin is answered next: the modular transplantation of the Gal-1 CRD onto Gal-3's N-terminal tail to create a Gal-3-like Gal-1 protein alters binding properties for these cell types relative to Gal-3 (Fig. 9g,h,l,m,q,r,v,w; Table 3). The special mode of presentation of photomicrographs in Fig. 10 enables the reader to spot described differences at a glance.

In summary, the approach of combining strategic engineering of galectin variants with their comparative testing has gained first insights into the ways the protein architecture determines the functionality of a CRD. On a fundamental level with relevance beyond galectins, it has thus proven its merits for clarifying architecture (protein design)-function relationships. The take-home message of this section is that the type of architecture

Table 3. Histochemical profiling of carbohydrate-inhibitable staining by biotinylated wild-type and variant galectins in sections of fixed adult murine testis[a].

Type of protein	Gal-1	$(Gal\text{-}1)_2$-GG	$(Gal\text{-}1)_2$-8S	$(Gal\text{-}1)_4$-GG	$(Gal\text{-}1)_4$-8S	Gal-3NT/1	Gal-3
Seminiferous tubules							
Spermatogonia[b]	++/+++	++	++	+	+	++/++	++
Spermatocytes[b]	++/+++	++/+++[c]	++/+++[c]	+/+++[c]	+/+++[c]	++/+++	++
Round spermatids[b]	++/+++	+++[d]	++++[d]	++++[d]	++++[d]	++/+++	++
Elongated spermatids[b]	+++	++++[e]	+++[e]	++++[e]	+++[e]	+++	+/++
Sertoli cells[b]	++/+++	++	++	+	+/-	++/+++	+/+++f
Basal lamina	-	-	-	-	-	-	-
Inter-tubular interstitium							
Leydig cells[b]	+++	-	-	-	-	+++	+
Vessel walls	+	-	-	-	-	+/-	-
Connective tissue	+	-	-	-	-	+	++
Macrophage-like cells[b]	-/+	+++	+++	+++	+++	-/+	+++

[a]intensity of staining in sections is grouped into the following categories: -, no staining; +, weak; ++, medium; +++, strong; ++++, very strong; [b]staining of cytoplasm; [c]Golgi region, <10% of cells; [d]Golgi region and caps (cap phase of acrosome formation, stage VI/VII of the spermatogenic cycle); [e]elongated acrosomal caps; [f]dot-like staining pattern of residual bodies (stage XII of the spermatogenic cycle); for details on protein characteristics, please see Kutzner et al., 2019.

(CRD display and modular design) is a key factor to shape the respective protein's activity profile as lectin. That a CRD is presented in more than one type of modular set-up, as shown in Fig. 8, enables functional diversity and so contributes to reach multifunctionality.

Conclusions

Nature's invention of high-density information coding by sugars is the molecular basis for a wide variety of (patho)physiological processes. This

Table 4. Five factors toward establishing multifunctionality of vertebrate galectins.

Factor	(Patho)physiological importance
Glycan specificity	ability to 'read' glycan-encoded signals with a β-galactoside core
Protein specificity	ability to bind distinct proteins (at the contact site for glycans or at different sites of the CRD as well as at other types of module in the chimera-type galectins or the linker between CRDs in tandem-repeat-type galectins)
Variability of protein architecture incl. modular design	ability to form different types of topological arrangements with counterreceptors, to alter quaternary structure by self-association and to build multi-contact complexes
Variability of gene (promoter, SNP[a]) and mRNA (alternative splicing[b]) sequences	ability to be expressed in specific spatiotemporal patterns with cell-type selectivity and responsiveness to inducers; ability to gain functional divergence by single-site deviations in gene sequences (in individuals) and by altering linker length by exon skipping in tandem-repeat-type galectins (e.g. exon V in CG-8)
Absence of a signal peptide	ability of galectins to first reside in the cytoplasm and also to then directly access the nucleus (requires development of export mechanisms to exert functions extracellularly besides release by cell death)

[a]single nucleotide polymorphism (e.g. F19Y in human Gal-8 or H64P in human Gal-3); [b]operative for the linker sequence of certain tandem-repeat-type galectins such as Gal-8.

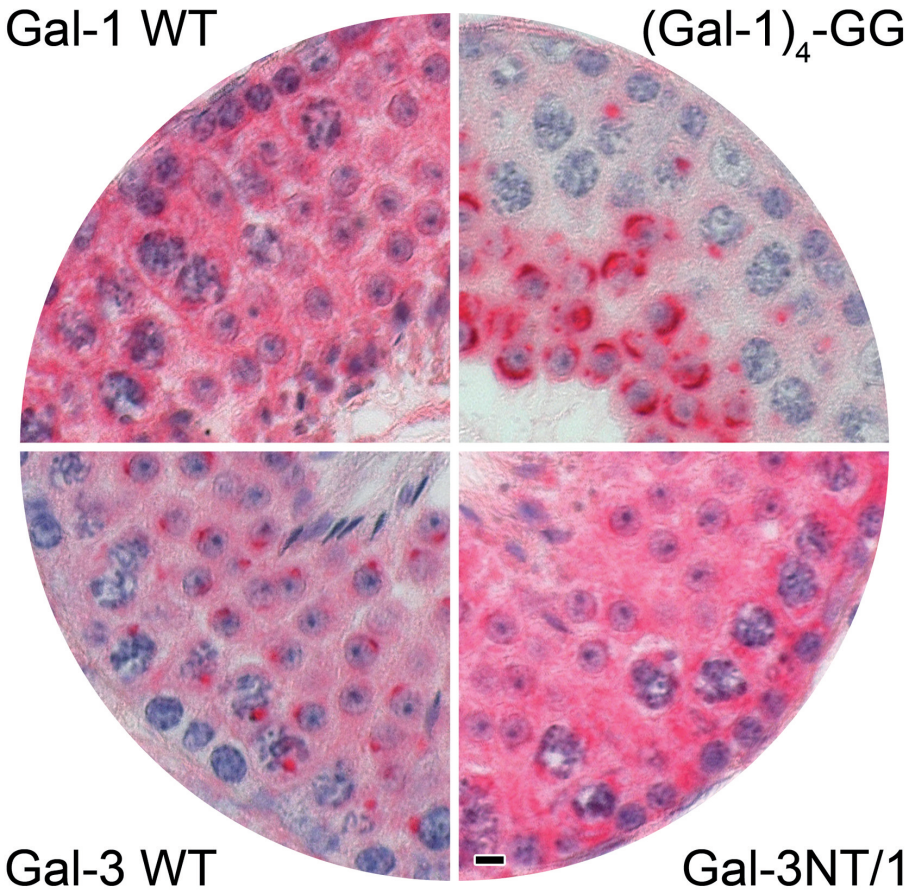

Fig. 10. Illustration of effect of altering galectin architecture on staining profiles of a seminiferous tubule of fixed adult murine testis. Wild-type (WT) Gal-1, its linker-connected tetrameric variant $(Gal\text{-}1)_4$-GG, Gal-3-like Gal-1 (Gal-3NT/1) and WT Gal-3 were tested on serial sections. Moderate and homogenously distributed staining of all cell types within the seminiferous tubule was characteristic for WT Gal-1 (top left). In contrast, the covalently linked tetramer selectively bound to the Golgi area of maturing spermatids (top right). The staining pattern of the Gal-3NT/1 variant (bottom right) with its strong positivity of the Golgi area and weak cytoplasmic staining of maturing spermatids was more similar to that of Gal-1 WT than to that of Gal-3 WT (bottom left). Probes (WT and variant galectins) were applied in the following concentrations: Gal-1 WT: 0.25 μg/mL; $(Gal\text{-}1)_4$-GG, Gal-3NT/1: 0.5 μg/mL; Gal-3 WT: 8.0 μg/mL. Scale bars: 5 μm.

perception guarantees future research on glycans a place in the limelight, drawing on a colloquial expression for work on lectins becoming more and more popular (Sharon, 1998). Since tissue lectins are a means to turn glycan-encoded signals into biological activities, increasing attention is in parallel naturally paid to characterizing their expression, structures and functions. That not a single but more than a dozen folds for proteins have all developed a contact region for glycans underscores the validity of the assumption of the fundamental significance of glycan-lectin recognition. Understanding this molecular interplay based on molecular complementarity means cracking the sugar code (Gabius, 2017). Matching the diversity and the dynamic regulation of the cellular glycome, ancestral lectin genes were the starting points to give rise to families of homologous proteins, by gene duplications and diversification with equally finely tuned expression profiles. This feature contributed to let lectins acquire multifunctionality. Factors that underlie multifunctionality have been identified and explained herein using galectins as instructive example.

To date, five characteristics are known to team up along the way toward multifunctionality for galectins (Table 4). As this table documents, there is much more to note than the capacities to bind glycans and to serve as a bridge between cells, a view initially held and already judged to be "restrictive" so that "it is certainly more full of promise" to rate galectin activities as being "dependent on the organ or cell type ...., and the same lectin may probably mediate several biological activities" (Caron et al., 1990). Henceforward, details obtained on each of the five listed items starting with the sugar specificity in Table 4 are sure to make their mark on the way we view (ga)lectins now and in the future.

The ongoing elucidation of how multifunctionality is attained in the case of galectins provides incentive to study other classes of vertebrate lectins accordingly, looking for analogies. Comparative considerations with plant lectins, using this information, are also proving worthwhile: leguminous lectins appear to help to achieve the dense packing of the contents of plant seed protein bodies by protein/protein-glycan interactions (Gansera et al., 1979; Einhoff et al., 1986; Freier and Rüdiger, 1987; Kummer and Rüdiger, 1988; Wenzel et al., 1993), exactly as suggested for vertebrate GRIFIN and its interplay with crystallins above. In both cases, a lectin is like a molecular glue (a term also applied when sugar determinants are involved to fabricate supramolecular entities, the "carbohydrate gluing" of the extracellular giant hemoglobin from the murine worm *Perinereis aibuhitensis* (Ebina et al., 1995; Yamaki et al., 1998)). It will thus not be surprising to find more cases of "gluing" with glycans and/or peptides as binding partner(s) for mammalian lectins.

Also, the emerging principle of teamwork between members of the galectin family, with potential for additivity/synergy or antagonism depending on the context and on the architecture of lectins, may well be extrapolated. Along this line, a productive interplay between members of various lectin families *in situ* can be envisioned. It can likely also be operative during dynamic glycan remodeling. The case study of GD1a-to-GM1 conversion illustrates the possibility for a mode of lectins working in relays: a receptor for ganglioside GD1a, i.e. myelin-associated glycoprotein (siglec-4), is known as inhibitor of axon growth by pairing with GD1a (Quarles, 2007), desialylation then abolishing this interaction and making Gal-1–GM1 binding possible for switching to neuritogenesis (Wu et al., 2016). In this sense, the information presented in this review is intended to offer inspiration and a guideline for further efforts to understand the fundamental rules of letting lectins become efficient and versatile translators of the sugar code.

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