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# Galectins in teleost fish: Zebrafish (*Danio rerio*) as a model species to address their biological roles in development and innate immunity*

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**Cell surface glycans, such as glycocoproteins and glycolipids, encode information that modulates interactions between cells, or between cells and the extracellular matrix, by specifically regulating the binding to cell surface-associated or soluble carbohydrate-binding receptors, such as lectins. Rapid modifications of exposed carbohydrate moieties by glycosidases and glycosyltransferases, and the equally dynamic patterns of expression of their receptors during early development, suggest that both play important roles during embryogenesis. Among a variety of biological roles, galectins have been proposed to mediate developmental processes, such as embryo implantation and myogenesis. However, the high functional "redundancy" of the galectin repertoire in mammals has hindered the rigorous characterization of their specific roles by gene knockout approaches in murine models. In recent years, the use of teleost fish as alternative models for addressing developmental questions in mammals has expanded dramatically, and we propose their use for the elucidation of biological roles of galectins in embryogenesis and innate immunity. All three major galectin types, proto, chimera, and tandem-repeat, are present in teleost fish, and phylogenetic topologies confirm the expected clustering with their mammalian orthologues. As a model organism, the zebrafish (*Danio rerio*) may help to overcome limitations imposed by the murine models because it offers substantial advantages: external fertilization, transparent embryos that develop rapidly *in vitro*, a diverse toolbox of established methods to manipulate early gene expression, a growing collection of mutations that affect early embryonic development, availability of cell lines, and most importantly, an apparently less diversified galectin repertoire.**
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***Keywords:*** **galectins, zebrafish, biological roles, development, innate immunity**

***Abbreviations:*** **ECM: extracellular matrix; Drgal1-L1, -L2, -L3: Galectin-1 like lectin 1, 2, 3, respectively, from *D. rerio*; Drgal3: Galectin-3 like lectin from *D. rerio*; Drgal9-L1: Galectin-9 like lectin from *D. rerio*; CRD: carbohydrate recognition domain; hpf: hour post fertilization.**

## Introduction

The diverse array of cell surface glycans, such as glycoproteins and glycolipids, contains rich structural information that is "decoded" by the binding of cell surface-associated or soluble carbohydrate-binding receptors [1,2]. This specific protein-carbohydrate recognition modulates interactions between cells, or between cells and the extracellular matrix. Further, and particularly during early development, the abovementioned structural information is subject to rapid changes mediated by glycosidases and glycosyltransferases [3]. Likewise, the decoding machinery of carbohydrate-binding proteins is also subject to change, and the dynamic expression patterns of such receptors in the context of early development suggest they play important roles in embryogenesis. Among these developmentally regulated carbohydrate binding receptors, galectins, a family of ß-galactoside-binding proteins, have been hypothesized to mediate fundamental processes in early development, but in most cases their specific functions and detailed mechanisms of action have yet to be rigorously demonstrated [4–6].

Studies on murine models suggest that galectins participate in embryo implantation in the endometrial surface, and myogenesis. During early stages of development, galectins-1 and -3

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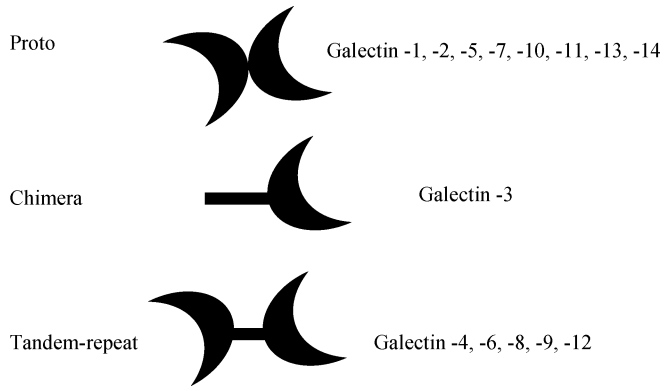


**Figure 1.** Classification of galectins. Based on subunit structure and molecular organization, galectins are classified in three basic types: proto, chimera, and tandem-repeat.

(see Figure 1 for classification) are differentially expressed and are believed to play a role in trophoblast implantation in the endometrium by binding nascent lactosaminoglycan ligands in the uterine wall [7]. Because galectin-1 is expressed prior to myoblast differentiation and binds to *N*-acetyllactosamine of laminin, it has also been proposed to play an important role in myoblast detachment/attachment [4]. The roles of galectin(s) in both developmental processes have yet to be established because null mutations for galectin genes in the mouse fail to produce phenotypic abnormalities [7]. A further screening indicated the presence of an additional galectin in the mouse embryo, galectin-5, which may mediate its implantation in the endometrial surface in the absence of galectins-1 and -3 [8]. This observation suggests that the lack of distinct phenotypes in the knockouts are due to the high "redundancy" associated with mammalian galectin genes: a total of fourteen distinct types of galectins have been described so far in higher vertebrates [9]. Such genetic "redundancy" makes it difficult to investigate the function(s) of galectin(s) in murine models by taking a gene knockout approach.

The zebrafish (*Danio rerio*) should constitute a suitable animal model for investigating biological roles of galectins in early embryo development because this species offers a number of advantages over mammalian systems. First, fertilization is external; the embryos develop rapidly *in vitro* and are transparent, making it possible to visualize the expression of putative genes mediating cell adhesion and/or migration. Second, the early expression of those gene(s) can easily be manipulated in zebrafish embryos, enabling functional studies of these genes. Third, a growing collection of interesting mutations that affect early embryonic development, such as cell adhesion and migration, are being generated and mapped to provide an excellent resource for future genetic studies of the function and mechanisms of actions of galectin(s) genes. Fourth, zebrafish cell lines have been established, which may be very useful to study gene expression and protein export at a cellular level. In this context, our preliminary investigations revealed the presence of four distinct galectins in zebrafish. Disruption of their expression resulted in phenotypes defective in somitogenesis and eye development.

## Galectins: General aspects

### Definition and classification

Galectins are an evolutionary conserved and ubiquitous group of lectins represented in most animal taxa examined so far, including the parazoa and both proto and deuterostome lineages, and fungi [5,9,10]. Two properties are required in a protein for its inclusion in the galectin family: (a) a characteristic carbohydrate recognition domain (CRD) with an affinity for ß-galactosides, and (b) a conserved sequence motif [4,5]. Based on structural features, galectins have been classified in three types: "proto", "chimera", and "tandem-repeat" (Figure 1) [11]. Prototype galectins contain one CRD per subunit, and are usually homodimers of non-covalently linked subunits. The C-terminal of the chimera-type galectins is similar to prototype, but its N-terminal contains a different gene product. Chimera type galectins are monomeric. Tandem-repeat galectins, in which two CRDs are joined by a linker peptide, are also monomeric. Proto and tandem-repeat types comprise several distinct galectin subtypes. Galectin subtypes have been numbered following the order of their discovery [12], and so far, fourteen have been described in mammals. Lower vertebrates and invertebrates appear to have a smaller galectin repertoire.

### Biochemical properties

From the biochemical, structural and genetic standpoints, galectins constitute one of the best-characterized lectin families [5,13–20]. Galectins are usually isolated by affinity chromatography on lactose-containing affinity resin column, and the biochemical properties, dimeric organization, and isoform diversity characterized for galectins from several sources [16,17]. Thermodynamic approaches have been used not only to assess the galectins' carbohydrate-binding properties, but also the oligomeric organization of the protein. On microcalorimetric studies, the dissociation constants for the interactions of bovine galectin-1 with the preferred ligands (lactose, *N*-acetyllactosamine, thiodigalactoside) were in the range of $10^{-5}$ M, with two binding sites per molecule [18]. Although galectin and legume lectins display a striking similarity in their 3-D structures, the thermal stability of the galectin is different from that of concanavalin A (Con A). Like Con A, the bovine galectin exists as a tetramer at the denaturation temperature, but, unlike Con A, it does not dissociate upon unfolding [18].

### Binding properties

#### *Carbohydrate specificity*

Although galectins are $\beta$-galactoside-binding lectins, the relative inhibitory activity of either D-galactose or its $\alpha/\beta$ methyl derivative is almost 200 times less effective than that of the

$\beta$-galactose-containing disaccharide lactose (Lac) as determined by several lectin binding assays [16,17,20–23]. For most galectins, *N*-acetyllactosamine (LacNAc) and thiodigalactoside (TDG) are 5 to 10 times more active than Lac. In general, 4′-OH, 6′-OH (Gal residue of Lac/LacNAc) are critical for binding, and thus, any changes (epimers or substitutions) of these hydroxyls reduce binding efficiency. The equatorial 3-OH of GlcNAc residue (on LacNAc) is also important for binding because Galß1-3GalNAc is a very poor inhibitor for most galectins. Substitutions of 2′-OH or 3′-OH or both (of Gal residue), however, do not affect the binding of galectin-1 to the carbohydrate ligand. Close examination of carbohydrate-binding specificities of galectins, however, revealed diversity in their binding properties [24]. Four oligosaccharides, Lac, LacNAc, Galß1-3GalNAc (T-disaccharide), and the human blood group A-tetrasaccharide were identified as useful tools to resolve the fine specificity of galectins, which could be further explained on a structural basis [24]. These significant differences in the galectins' carbohydrate specificities, and the conservation of amino acid residues that interact with the carbohydrate ligands, enabled the classification of galectins' CRDs into two types, "conserved" or "variable" CRDs [24]. For example, *Bufo arenarum* galectin shows specificity similar to bovine galectin-1 probably due to similar structures of their binding sites, but the specificity profile of 16 kDa galectin from *Caenorhabditis elegans* with the four abovementioned carbohydrates is different from those observed for the bovine and amphibian galectins [23]. The *C. elegans* 16 kDa galectin showed a novel carbohydrate specificity pattern that has not been described in any of the known galectins characterized so far [23]. The binding profiles of these galectins have been explained by the analysis of their 3-D structures [19].

### *Endogenous ligands*

Glycoproteins or glycoconjugates that contain polylactosamine chains have been proposed as the most likely endogenous ligands for galectins. Laminin and lysosome-associated membrane proteins have been shown to interact with galectin-1 [25–27]. Mucin and fibronectin are also good ligands for the avian and amphibian galectins [28,29]. Galectin-3 interacts with IgE and its receptor [30], laminin and fibronectin [31], and mucin [32]. The biological function of a particular galectin, however, may vary from site to site, depending on the availability of suitable ligands [4]. Nevertheless, the nature of the endogenous ligands remains largely unknown for most lower vertebrates and invertebrates.

## Protein structure and gene organization

### *Primary structure*

Galectins lack typical secretion signal peptides [11]. The N-terminal amino acid residue in most galectins is blocked by acetylation [4,9]. Galectins are not glycosylated, although some of them have a potential glycosylation signal. The primary structures of galectins-1 from mammals are substantially conserved (87–90% amino acid identity with human galectin-1) [5,11]. Percentage identities of human galectin-1, when aligned with galectins from the non-mammalian taxa, are generally lower: 55 to 57% with two galectins from chicken; 33 to 45% with galectins from ectothermic vertebrates [*B. arenarum* (45%), *Xenopus laevis* (40%), electric eel (42%) and conger eel (33%)], and 21 to 27% with galectins from invertebrates, [*C. elegans* (25-27%) and *Geodia cydonium* (21%)]. The N-and C-terminal regions of the molecule are not as highly conserved as the CRD. The CRD comprises a stretch of 30 amino acids, and shows higher identity relative to the whole peptide, even among galectin types. For the chimera type galectins (galectin-3), the two domains are 27 to 29% homologous to that of human galectin-1, whereas their CRDs show about 57% identity with that of human galectin-1.

### *Gene organization*

The gene organization of galectins from human, mouse, and chicken is remarkably similar. Two galectins-1 from human and mouse [33,34], galectin-2 [35] and chicken 14 kDa galectin [36] have identical numbers of exons, with similar size. Each gene (about 3.2 kb) contains four exons (from the 5′ end to the 3′ end: exon I, 6 to 9 bp; II, 80 to 83; III, 160 to 172 bp; and IV, 144 to 150 bp) of which the largest one (exon III) encodes the CRD. The gene structures of galectin-3 from mouse and rat show that the exon-intron structure of their CRD domain is similar to the 14 kDa galectins, whereas their N-terminal domains are encoded by two exons [37]. The genomic structure of the *C. elegans* 32 kDa galectin revealed a unique intron organization: two introns of which the first one is conserved with mammalian galectins [38].

### *Structural basis of lectin-carbohydrate interactions*

The structures of mammalian galectins-1, -2, -3 (C-terminal), -7, and -10, and the galectin from *B. arenarum* are quite similar [15,19,39–42]. The structure shows a jellyroll topology typical of legume lectins (Figure 2). The subunit of galectin is composed of an 11-strand antiparallel ß-sandwich and contains one CRD. In the dimer, the N- and C-terminal are at the dimer interface. The 3-D structure of the bovine galectin-1-*N*-acetyllactosamine complex enables the identification of the amino acid residues that participate in interaction with the ligands, as well as the position and orientation of the hydroxyls from the sugar that are interacting with those amino acids [19]. The overall results from the 3-D structure were consistent with the studies on carbohydrate-binding specificities of galectins [16,17,21,22]. Interestingly, multiple galectin-1 isoforms co-crystallize in the galectin-ligand complex (Ahmed and Vasta, submitted).

## Evolutionary aspects

Comparisons of the primary structures of galectins from various species indicate that this protein family is evolutionarily

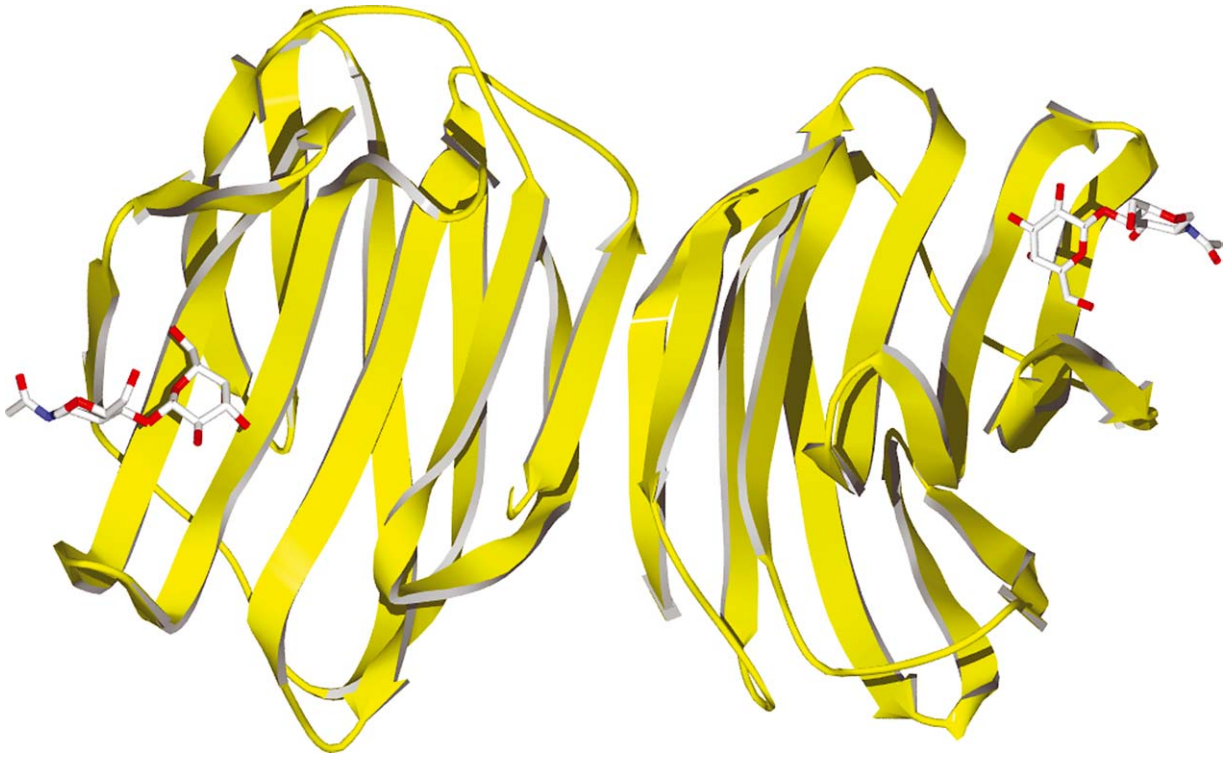

**Figure 2.** Structure of the galectin-1/*N*-acetyllactosamine complex. Galectin-1 is a dimer displaying a sugar-binding cleft at each end, with each subunit organized as 11-strand antiparallel ß-sandwich (see references 15 and 19).

conserved. The presence of galectins in the sponge *G. cydonium* [43] and the fungus *Coprinus cinereus* [44] reveals their early emergence and structural conservation in eukaryotic evolution, and supports the notion that they mediate fundamental mechanisms in cell to cell interactions or other functions. Hirabayashi and Kasai [11] found a linear correlation for the galectin sequence homology and the phylogenetic distance between the source species and proposed constant mutation rates. For example, assuming the rate of amino acid substitution is constant during evolution, the time of divergence of nematode and sponge were estimated to be 680 and 800 million years ago, apparently in good agreement with the generally accepted time of divergence for those taxa. However, *B. arenarum* galectin is closer, both in primary structure and carbohydrate specificity, to the bovine galectin-1 than to the galectin from a related amphibian species, *X. laevis,* indicating that, within this lectin family, structural and functional divergence may have occurred early in vertebrate evolution [16]. A likely explanation for this observation may be that mutation rates would not be constant within the galectin family and the galectin-1 subgroup may have evolved under low mutation rates. If this is the case, the galectin-1 would be a considerably homogeneous category within this lectin family, and the high conservation of residues in the Type I CRD that interact directly with the ligand relative to those in the Type II CRD [24], would buttress this idea. However, recent studies indicated that the *X. laevis* contains multiple galectin-1 like proteins that are closer to *B. arenarum* galectin in their primary structures [45, Ahmed and Vasta, unpublished].

Galectins from invertebrates are more divergent among them and from those of mammals both in primary structure and gene organization. Although the exon-intron organization is conserved in the galectins examined so far, the *C. elegans* tandem-repeat galectin gene possess an intron (intron II), which is not conserved in mammalian galectins [38]. However, the insertion pattern of this intron is common to several *C. elegans* tandem-repeat galectins, suggesting that they are the product of a gene duplication event in the protostome lineage, which paralleled that in deuterostomes [38].

## Subcellular localization and secretion

Although galectins lack a typical secretion signal peptide [9,11], they are present not only in the cytosol but also in the extracellular matrix [46,47]. From the cytosol, galectins may be targeted for secretion by non-classical mechanisms, possibly direct translocation across the plasma membrane [46,48]. In cultured muscle cells galectin-1 is externalized during differentiation, apparently by membrane evagination [46]. In filter-grown confluent monolayers of Madin-Darby canine kidney cells, galectin is secreted from the apical domain of the polarized cells, whereas laminin is secreted from the basal domain and becomes incorporated into the matrix between cells and

substratum [49]. Galectins may also be translocated into the nucleus [50], into vesicles [51], or accumulate at sub-cytosolic sites [52]. Further, the cellular localization of galectins varies at different developmental stages, as observed in chicken retinal cells [53]. Striking differences in the distribution of galectins have been described for other cell types. For example, in the liver, lectin was found concentrated in the lining of hepatic sinusoids [54], whereas in the pancreas it was localized extracellularly [54]. In the skeletal muscle, the lectin was detected both on the surface and within myoblasts [55], whereas in the kidney it was detected extracellularly and was never found intracellularly [56].

## Biological roles

Galectins have been proposed to mediate diverse biological processes such as embryogenesis [7,25], inflammation [57], apoptosis [58], neuron projection [59], tumor metastasis [60], and B-cell development [61]. However, rigorous demonstration of these roles *in vivo* and the detailed mechanism of these functions have remained elusive due largely to problems inherent to the mammalian models used. For example, the proposed biological functions of galectin-1 in mediating cell to cell and cell to extracellular matrix interactions have been difficult to reconcile with the original observation that in fibroblasts the lectin is found primarily in the cytoplasm, and in the absence of reducing agents the isolated lectin is readily oxidized and inactivated [62]. Galectin-1 contains six free cysteine residues, which outside a reducing environment, may form disulfide bonds [63]. Because other mammalian galectins and galectins from ectothermic vertebrates and invertebrates lack the conserved cysteine residues, binding properties and biological functions of galectins may not be dependent on their oxidation state [24]. Furthermore, when associated with their ligands, such as lactose or laminin, galectins remain active in oxidating environments [16,64].

### *Functions in early development*

The dynamic patterns of expression of galectin-1 and galectin-3 during mouse embryogenesis led to propose their potential roles at several developmental stages such as notochord development, somitogenesis, and development of central nervous system [7,65]. However, mice carrying null mutations in these galectins (double knockout) do not exhibit any overt phenotype [8]. A further screening indicated the presence of an additional galectin in the mouse embryo, galectin-5, which may compensate the biological function in the absence of galectins-1 and -3 [8]. Thus, the effects of null mutations on normal development are not drastic, probably due to functional redundancy of galectins during development [8]. At the present time, fourteen distinct types of galectins have been described in mammals (see Figure 1), and additional, as yet undescribed galectin types are evident from GenBank databases [9]. Nonetheless, subtle phenotypes have been identified in mice [59,66]. In galectin-1 null mice, a group of primary sensory neurons failed to project their axons to the correct sites in the caudal olfactory bulb suggesting that galectin-1 may be involved in neuronal path-finding and neurite outgrowth [59]. Galectin-3 is expressed at the onset of chondrification suggesting a role in bone development [66]. This is supported by the finding that galectin-3 null mice show abnormalities in cells of the hypertrophic zones, and a reduction in the total number of hypertrophic chondrocytes [66]. However, the considerable diversity and potential functional "redundancy" of the galectin repertoire, makes it difficult to investigate the function(s) of galectin(s) in murine models by taking a gene knockout approach.

### *Functions in innate and adaptive immunity*

There is a fragmentary, albeit significant, body of evidence that galectins participate both in innate immunity and developmental aspects of adaptive immunity. Galectin-1 participates in acute and allergic inflammation [57]. Galectin-1 ameliorates edema induced by bee venom phospholipase A2 [67] and inhibits the release of arachidonic acid from LPS-stimulated macrophages, neutrophil extravasation and mast cell degranulation [67]. Galectin-1 influences the ability of macrophages to control intracellular infections either by inhibiting microbicidal activity or inducing host-cell apoptosis [57]. In *Trypanosoma cruzi*-infected macrophages, galectin-1 blocks IL-12 production, thereby promoting parasite replication [68]. Galectin-3 is normally expressed in epithelia of many organs and various inflammatory cells, such as macrophages, dendritic cells and Kupffer cells [69]. In contrast to the pro-apoptotic activity of galectin-1, galectin-3 has an anti-apoptotic activity [70]. This has been supported from studies of galectin-3-deficient mice since they undergo accelerated apoptosis after treatment with apoptotic stimuli [71]. In contrast to the anti-inflammatory effects of galectin-1, galectin-3 shows pro-inflammatory activity. The expression of this lectin is upregulated during inflammation, cell proliferation, and cell differentiation [69]. Galectin-3 activates NADPH oxidase [83], stimulates superoxide production from neutrophils [73], potentiates LPS-induced IL-1 production, and promotes monocyte chemotaxis [69]. The pro-inflammatory role of galectin-3 has been supported from studies of galectin-3 knockout mice, as they had fewer inflammatory cells with reduced levels of NFkB activation after an intraperitoneal challenge [71]. Galectin-9, a tandem-repeat galectin, and a selective chemoattractant for eosinophils [74], is highly expressed in tissues of the immune system, such as bone marrow, spleen, thymus and lymph nodes [74]. Galectins-10 and -14 are selectively expressed in eosinophils, suggesting their roles in allergic processes [75].

With regard to adaptive immune functions, galectins and their ligands have been proposed as regulators of immune cell homeostasis [57]. Galectin-1 inhibits proliferation of mitogen-activated T cells and reduces clonal expansion of antigen-primed $CD8^+$ T cells and human leukemia T cells in a carbohydrate-dependent manner [76]. Galectin-1 induces

apoptosis of human and murine T cells during development in the thymus [58]. Apoptosis of thymocytes and T cells is modulated by CD45 and CD7, of which the latter is critical [58,77]. Galectin-1 expressed by bone marrow stromal cells has been demonstrated to mediate maturation of B-cells by binding to the surrogate light chain of the preB cell receptor [61].

*Galectins in teleost fish: Current knowledge*

Electrolectin, the first galectin identified and characterized, was isolated from the electric organs of the electric eel *Electrophorus electricus* [78]. Since then, a substantial number of galectins have been identified in elasmobranch and teleost fish, and characterized in various degrees of detail (Table 1).

*Isolation, biochemical characterization, and tissue localization.* Characterization of electrolectin by reducing SDS-PAGE, amino acid analysis, and sedimentation equilibrium [78], revealed a globular, relatively hydrophilic protein with subunit weight of ~16,500 Daltons that forms a non-covalent dimer in the presence of ligand (Table 1). Though electrolectin possesses no cysteines, it is sensitive to inactivation by oxidation, as measured by hemagglutination activity. Comparison of its amino acid sequence with galectins from human and chicken revealed that it is a member of the galectin family [79]. Although galectins are usually of wide tissue distribution, the presence of galectins in skin and gut mucus of fish has been particularly intriguing with regard to their potential biological role(s) in defense against microbial pathogens. Two prototype galectins from skin mucus of the conger eel *Conger myriaster* (congerins: 13 kDa subunit, dimer in native form) [80] not only agglutinate red blood cells from different species but also the fish pathogen *Vibrio anguillarum*. The amino acid sequence of one congerin (Con I) [81] revealed a 135 amino-acid subunit homologous to electrolectin and mammalian prototype galectins. The sequence of the second congerin (Con II) [82] revealed that although of equal subunit length, it is only 46% homologous to Con I, and differs in thermostability, pH dependency for hemagglutinating activity, and fine carbohydrate specificity. Immunohistochemistry indicated that congerins are only present in the epidermis of the skin and lining of the alimentary tract preceding the stomach [83]. The subunit structures of Con I and II are similar, but they differ in the dimer interfaces [84,85], thus explaining the differences in thermal stability and carbohydrate specificity observed. Similarly, a 16 kDa galectin (AJL-1) was isolated from the skin mucus of the Japanese eel, *Anguilla japonica,* by lactose affinity chromatography. This 142 amino-acid protein is N-terminally blocked, consists of two identical subunits, and has no disulfide bonds. A prototype galectin, also present in skin mucus, was purified and partially characterized from skin and muscle of the striped bass *Morone saxatilis* (Henrikson, Ahmed, and Vasta, unpublished). It has a subunit size of 15 kDa, N-terminally blocked, and forms a homodimer under non-reducing conditions. It is ubiquitous in the animal tissues, with the exception of the plasma. Its genomic organization is the same as mammalian and chicken prototype galectins, varying only in intron size.

*Structural relationships with galectins from mammals, amphibians, and invertebrates.* In addition to amino acid or gene sequences of the fish galectins described above, a substantial number of galectin sequences from teleost fish are available from GenBank. These include prototype galectins from flounder (*Paralichthys olivaceus*) and medaka (*Oryzias latipes*), prototype and tandem repeat galectins from Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*), and a possible tandem repeat galectin from catfish (*Ictalurus punctatus*). To date, there are nine open reading frames that code for $\beta$-galactoside-binding CRD's in the Japanese pufferfish (*Takifugu rubripes*). Two are linked to another, quite similar to tandem repeat galectins found elsewhere. The remaining five exhibit varying levels of identity with mammalian galectin-1. Genomic sequence and annotation based on sequence identity is the extent of *T. rubripes* galectin characterization. From the green spotted pufferfish (*Tetraodon nigroviridis*) a contiguous sequence that contains an entire prototype galectin gene is similar to the *T. rubripes* and *M. saxatilis* prototype galectins. The gene organization of *T. nigrovirdis* galectin is similar to that of *T. rubripes*, mouse and human galectin-1, and chicken-16. Several galectin sequences have been identified from our studies and the zebrafish (*D. rerio*) genome database, including members of the three galectin subtypes, proto, chimera and tandem-repeat [20], and will be discussed separately in a later section.

Alignment of the abovementioned amino acid sequences (either authentic or deduced from cDNAs) revealed that these lectins are members of the galectin family (Figure 3). Based on their subunit molecular size, most of them belong to the prototype galectin subgroup, except for the trout galectin 9, and zebrafish Drgal3, Drgal9-L1. If only the CRD region (shaded amino acids in Figure 3) is compared, Drgal1-L1, -L2, -L3, *E. electricus, F. rubripes, T. nigroviridis,* and *P. olivaceous* are more closely related to the galectin-1 group than to any other galectin family members. Percentage identities to the human galectin-1 vary from 22% (domain I of Drgal9-L1) to 42% (Drgal1-L3). Among the fish galectins, the highest identity observed is between galectins from *T. rubripes* and *T. nigroviridis*. Within the three prototype galectins from zebrafish, Drgal1L1 is 79% identical to Drgal1-L2 but only 45% to Drgal1-L3. The phylogenetic topologies of galectins from teleost fish and selected representatives of other vertebrate taxa were examined unrooted dendrograms (Figures 4 and 5) generated using the ClustalW program, in which the neighbor-joining method of Saitou and Nei [86], is applied to a distance matrix generated by calculating the percent divergence between all pairs of sequences from a multiple alignment. Although there is a close relationship among all of vertebrate prototype galectins (Figure 4), the teleost galectins are removed from the tight group formed by mammals, birds, and amphibians, and the prototype galectins

**Table 1.** Summary of teleost galectins

| | *Protein name* | *Type* | *Length* | *DNA submitted* | *Gene structure* | *Protein purified* | *Protein character.* | *Amino acid submission* | *Primary structure* | *MW (calc)* | *% identity human galectin-1* | *Genome size (Mb)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Conger myriaster* (I) | Congerin I | Proto | 135 | AB010277 | No | Yes | Yes | Yes P26788 | Yes | 15,448 | 32 | 1220 |
| *Conger myriaster* (II) | Congerin II | Proto | 136 | AB010276 | No | Yes | Yes | Yes Q9YIC2 | Yes | 15,379 | 30 | 1220 |
| *Anguilla japonica* | AJL-1 | Proto | 142 | AB098064 | No | Yes | No | Yes BAC67210 | Yes | 16,091 | 27 | 1400 |
| *Electrophorus electricus* | Electrolectin | Proto | 136 | No | No | Yes | Yes | Yes P08520 | Yes | 15,467 | 42 | ? |
| *Morone saxatilis* | MS-15 | Proto | 135 | No | Yes | Yes | Yes | No | Yes | 14,931 | 40 | 890 |
| *Danio rerio* | Drgal1-L1 | Proto | 135 | AW174841 | Yes | Yes | Yes | No | Yes | 15,556 | 39 | 1700 |
| *Danio rerio* | Drgal1-L2 | Proto | 134 | AY421704 | Yes | Yes | Yes | Yes AY421704 | Yes | 15,264 | 36 | 1700 |
| *Danio rerio* | Drgal1-L3 | Proto | 132 | Dr15088 | Yes | Yes | Yes | No | Yes | 14,803 | 42 | 1700 |
| *Takifugu rubripes* | None | Proto | 131 | Yes | Yes | No | No | No | No | 14,848 | 36 | 400 |
| *Tetraodon nigroviridis* | None | Proto | 134 | Yes | Yes | No | No | No | No | 15,010 | 40 | 510 |
| *Paralycthys olivaceus* | None | Proto | 135 | Yes | No | No | No | No | Yes | 15,384 | 37 | 710 |
| *Oryzias latipes (I)* | None | Proto | inc | TIGR BJ495563 | No | No | No | No | No | incom | 31 | 700 |
| *Gasterosteus aculeatus* | None | Proto | >129 | Yes | No | No | No | No | No | incom | 37 | 700 |
| *Ictalurus punctatus (I)* | None | Proto | 132 | CF261531 | No | No | No | No | No | 14,863 | 34 | 1000 |
| *Salmo salar* | None | Proto | 129 | No | No | No | No | No | No | 14,725 | 38 | 3000 |
| *Oncorhynchus mykiss* | None | Proto | 129 | No | No | No | No | No | No | 14,758 | 37 | 2600 |


**Figure 3.** Amino acid sequence comparison of fish galectins with galectins from vertebrates. Amino acid sequences are aligned by ClustalW program. The amino acids (shown in light shaded) are known to interact with *N*-acetyllactosamine as determined from 3-D structure of the bovine galectin-1 [15]. The amino acids in congerin II (shown in dark shaded) are believed to constitute extended binding site [85].

of *D. rerio* cluster with those from teleosts. Specifically, of the three *D. rerio* prototype galectins so far identified, two cluster together with the freshwater knifefish (Drgal1-L1 and -L2), with an overall 59% degree of identity with electrolectin, while the third (Drgal1-L3) clusters with the anadromous salmonids with an overall 60% degree of identity with *O. mykiss* and *S. salar* prototype galectins. This phylogenetic analysis of prototype galectins of teleosts match the current taxonomy to the level of order in most of the species analyzed. The Order Anguilliformes groups together as do the Superorder Percomorpha and the Order Salmoniformes. *D. rerio* is classified within the Order Cyprinoformes, and Drgal1-L1 and Drgal1L2 groups with

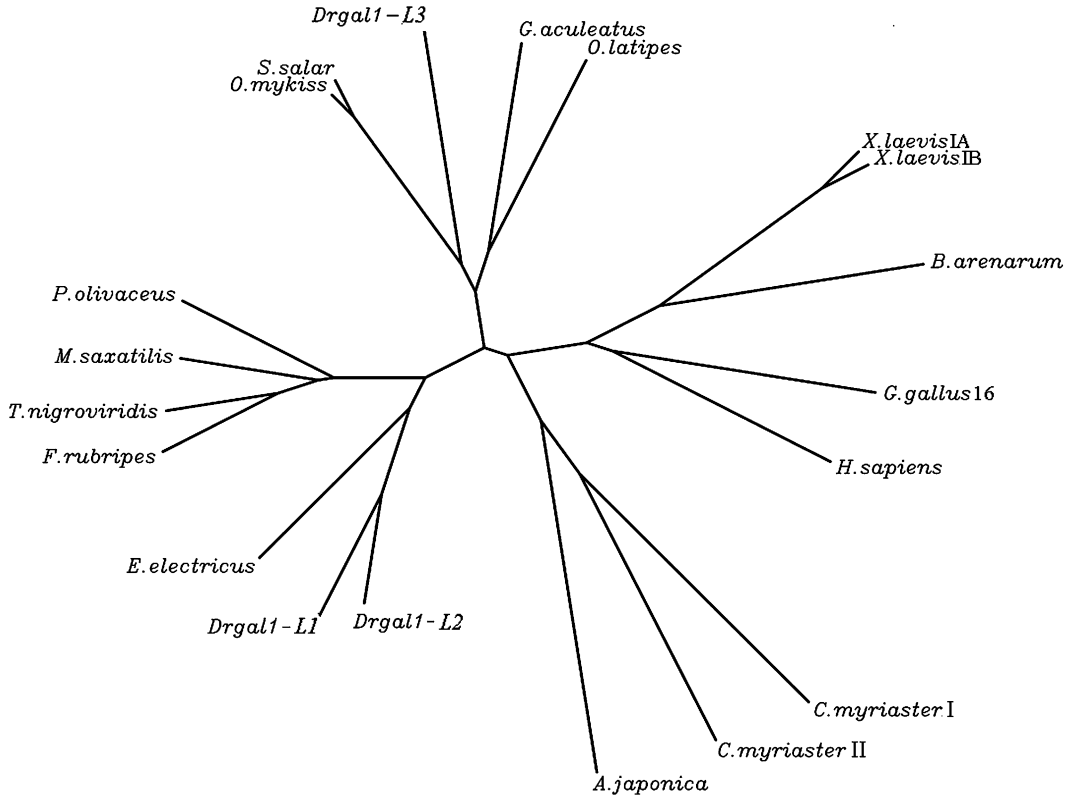


**Figure 4.** Relationship of *D. rerio* prototype galectins to the teleost prototype galectins. The dendrogram shows the relationships of the complete sequences of twelve teleost fish prototype galectins to the human galectin-1, chicken 16, *B. arenarum* galectin-1 and two *X. laevis* prototype galectins. This was generated using the computer program ClustalW (clustalw.genome.ad.jp/).

the Order Gymnotiformes (electrolectin), a sister group of the Cyprinoformes. Drgal1-L3, however, groups with galectins of medaka and stickleback (both Percomorpha), and with those from trout and salmon (both Salmoniformes). As in *D. rerio,* it is likely that multiple prototype galectins are present in these species, and thus, the sequences analyzed are the products of orthologous and paralogous genes.

### *Teleost fish species as models for developmental and genomic studies*

In recent years, a small number of teleost fish, such as zebrafish (*D. rerio*) and medaka (*Oryzias latipes*), have been established as models for developmental studies in morphogenesis, neurobiology, and immunity. Many orthologous genes are shared among fish and mammals, a significant advantage over more phylogenetically-distant models such as *Drosophila* and *C. elegans,* which lack genes involved in many functions typical of vertebrates [87]. Although genomic databases for the identification and analysis of genes of interest are currently under development for the abovementioned species [88], the relatively small genomes of pufferfish (*T. rubripes* and *T. nigroviridis*) have supported their use as models of choice for genomic initiatives [89].

Recent advances in the cell biology, and technical manipulations of zebrafish are making this model increasingly more attractive for developmental and immunobiological studies [87].

Further, the potential of the zebrafish EST and genome projects is substantial, but identification of orthologues of the mammalian counterparts may require caution. Because it is believed that a genome duplication event took place after the divergence of teleost fish from the mammalian lineage, it remains unclear how much of the hypothetical duplicated genome is present in zebrafish [87]. If both orthologues are present, structural and functional divergence should be considered because the duplicated genes would tolerate a higher rate of mutation in sequence and function [87]. For the zebrafish galectins, this consideration does not appear to constitute a problem as its galectin repertoire appears far less diversified than in human, thus facilitating functional analysis of the individual components. In comparison with other vertebrate genomes, *T. rubripes* has one of the smallest genomes, comprising approximately 400 Mb [89]. The pufferfish genome contains less repetitive DNA sequences, smaller intergenic regions, and smaller introns (The Fugu Landmark Mapping Project). As an established model vertebrate genome, it is an economical tool on which comparative sequence analyses can be performed. Two teleost sequencing projects, one for *T. rupribes* (International Fugu Genome Consortium) and one for *T. nigroviridis* (Center for Genome

Research) are currently underway. Although with longer generation time than zebrafish, medaka has also been suitable for use in genomic (small genome size) and developmental studies (transparent embryos) [90].

## Zebrafish as a model species for elucidating the biological roles of galectins

### *Studies on early development*

The use of zebrafish as a model for addressing developmental questions in higher vertebrates, including mammals, has expanded dramatically in the past few years [88,91,92]. The popularity of this animal model can be explained by the advantages it offers over mammalian systems [91,92]. First, fertilization is external, the embryos develop rapidly *in vitro* and are transparent, making it possible to visualize the effects of genes involved in developmental processes, such as cell migration, organogenesis, etc. Second, the early expression of these gene(s) can be easily up- or down-regulated in zebrafish embryos, enabling the analysis of the effects of their normal or experimentally modified expression. Third, a growing collection of mutations that affect early embryonic development have been characterized and mapped, providing a powerful resource for genetic studies on the function and mechanisms of action of developmentally-regulated genes.

### Studies on immunity during embryogenesis

In addition to its usefulness as a model for genetics and development, recent studies support the notion that zebrafish can be an equally attractive model for addressing fundamental questions in immunobiology [87,93–97]. Several significant advantages over mammalian models are offered by the zebrafish model, that can advance our understanding of the immune system: (a) because of the embryo development ex-utero and transparency, morphological and cellular aspects, such as development of lymphoid tissues and inflammatory cellular responses, can be directly examined and potentially manipulated at far earlier points in development; (b) mutations and screening for mutant phenotypes can be achieved on a larger scale, faster and in a more cost-effective manner than other systems; (c) transgenesis, for example dominant negative approaches, can be achieved more efficiently and may be more informative for developmental studies of the immune system; (d) additional approaches, such as the use of antisense knockdowns for the genes under study can be very useful for characterizing their roles in early embryogenesis [87].

#### *Innate immunity*

Because in zebrafish no components of adaptive immunity (T or B cells, or expression of genes characteristic of adaptive immunity (*rag* 1 and 2, *Ig*) can be detected before 4 days post-hatching [93], it can be assumed that until that time the embryo relies heavily in innate immune mechanisms to fight infection. Among humoral components of innate immunity, the zebrafish exhibits cytokines and interferon [98,99], and pathogen-recognition molecules such as F-type lectins [100, Odom and Vasta, in preparation]. With regard to cellular responses, the zebrafish transparent free-living embryo is ideal for investigating early stages of lymphopoiesis and inflammation during early embryogenesis. Cells that constitute hallmarks of innate immunity, such as granulocytes and macrophages, have been identified and functionally characterized in zebrafish. Like mammals, zebrafish possess neutrophils and eosinophils [95]. These two distinct granulocytes and macrophages circulate in blood as early as 48 h post fertilization (hpf) [95]. Embryonic macrophages are actively phagocytic and can remove carbon particles from the circulation [95], phagocytose apoptotic corpses, and engulf and destroy large amounts of bacteria injected intravenously [94]. Both macrophages and granulocytes accumulate at inflammation sites in experimental wounds [95], and in experimental mycobacterial infections can form granulomas typical of mammalian models [96]. Molecular markers such as myeloperoxidase for granulocytes [95], and draculin and leucocyte-specific plastin for macrophages [94] have been identified, cloned, and used to study myelopoiesis and inflammatory responses in zebrafish early embryogenesis, indicating that at 48 hpf embryonic granulocytes and macrophages are functionally active [94,95]. Furthermore it has been shown that although only a fraction of the macrophage population goes to the site of infection, the entire population acquires an activated behavior similar to that of activated to that of macrophages in mammals [94].

#### *Adaptive immunity*

Like with inflammatory responses, several features of the zebrafish adaptive immune system resemble those of higher vertebrates, including thymus organization and ultrastructure, germ line and somatic diversity of Igs, general aspects of MHC, and patterns of expression of *rag*1 and *rag*2 genes [87]. Moreover, although only TCR$\alpha$ has been described in zebrafish, from evidence in related taxa (elasmobranchs) it is assumed that all four TCR isotypes are present in zebrafish. Furthermore, sid1, which encodes a secreted immunoglobulin domain and shares structural properties with VpreB, a surrogate light chain that functions in early stages of B-cell receptor expression, has been identified in zebrafish [87]. In mammals, interactions of the surrogate chain in preB cells with galectin modulate maturation of B cells in bone marrow [61].

#### *Experimental infection and disease*

The well-developed adaptive and innate cellular immune systems of zebrafish support its use as an attractive model for the study of infectious diseases [97]. Infections by Gram-positive and Gram-negative bacteria, fungal (*L. mutabilis*) infections, and parasites such as nematodes, microsporidians, and dinoflagellates, have been associated with disease in zebrafish [87].

Experimental models for infectious disease by *Streptococcus* sp. and *Mycobacterium* sp. have been recently developed and characterized [96,97]. Following *Streptococcus* injection, zebrafish develop infections resembling not only those observed in farmed fish populations, but also human streptococcal diseases [97]. Like in human tuberculosis, infection of zebrafish with pathogenic mycobacteria produces granulomas, highly organized structures containing differentiated macrophages and lymphocytes that sequester the pathogen. The transparency of zebrafish embryos, which in early stages have macrophages and granulocytes but lack lymphocytes, allows to directly visualize the events of mycobacterial infection and formation of granulomas *in vivo*, solely in the context of innate immunity [96]. Galectins have been proposed to mediate multiple roles in innate and adaptive immunity. However, adaptive immunity genes are expressed only after 4 days post-hatching. Therefore, the zebrafish model should constitute an ideal model to dissect the functions of galectins before and after adaptive immunity becomes active in resistance to disease. As the study of galectins in experimental models of infection are developed, mutagenesis screens can be created to examine the genetics of galectin-mediated disease resistance and susceptibility.

### Zebrafish cell lines

Various somatic cell lines (ZEM2S, ZF4, ZFL, SJD.1, AB.9) have been recently established, and are available from American Type Culture Collection (ATCC), Manassas, VA. These constitute useful tools for studying the expression of gene of interest and protein export at a cellular level. The cell lines ZEM2S [101] and ZF4 [102] were established from zebrafish embryos (blastula and 1-day old, respectively). The ZF4 cell line was derived from adult liver [103]. The SJD.1 and AB.9 are fibroblast cell lines derived from caudal fins of an adult zebrafish from strain SJD and AB, respectively [104]. In the presence of cells from a rainbow trout spleen cell line (RTS34st), zebrafish embryo cells were able to produce germ-line chimeras when introduced into a host embryo [105].

## Galectins in zebrafish

### Current knowledge

To address questions related to the biological roles of galectins in embryonic development and immune process, we recently initiated studies on the zebrafish model [20].

#### *Diversity of the galectin repertoire*

By using various approaches (protein purification and characterization, cloning, and in silico data mining), we have identified and characterized the zebrafish galectin repertoire: three prototype galectins (Drgal1-L1, Drgal1-L2, Drgal1-L3), one chimera type galectin (Drgal3), and two tandem-repeat type galectins (Drgal9L1, Drgal9-L2) [20] (see Table 2). All zebrafish galectins characterized showed remarkable structural similarities with mammalian galectins, and this enabled their unambiguous classification within the three well-established galectin groups. Galectin repertoires of protostome invertebrates and lower vertebrates appear to be limited to one or two galectin types [9]. For example, *C. elegans* and *Drosophila* have proto and tandem-repeat type galectins [9,38,106]. In this aspect, it is noteworthy that at the evolutionary level of teleosts, all three galectin groups (proto, chimera, and tandem-repeat) are already represented. Nevertheless, based on our studies and the information currently available from the zebrafish genome project, it seems quite clear that the zebrafish galectin repertoire is smaller than that of mammals. Therefore, this species should be a more suitable model organism for the study of biological roles of galectins, than the mammalian model systems where fourteen distinct galectins have been already identified [9], and additional galectin members are evident from GenBank databases [9]. In Figure 5, the *D. rerio* galectin types can be identified by their clustering with the known galectin groups. This information suggests that a diverse galectin repertoire was present early in vertebrate evolution, and that the structural and perhaps functional properties of these galectins have been highly conserved over a period of ~400 million years, which is the evolutionary distance between teleost fish and modern mammals. Thus, although the teleost galectins cluster separately from the terrestrial tetrapods (Figure 4), it is clear that the prototype galectins all cluster more closely than do the chimera type and the tandem-repeat type galectins.

#### *Structural similarities with mammalian galectins*

Homology modeling of Drgal1-L2 [SWISS-MODEL, Version 36.0003 [107] at the SWISS-MODEL Protein Modeling Server (http://swissmodel.expasy.org)] based on the bovine (*Bos taurus*) spleen galectin/*N*-acetyllactosamine complex structure [15], revealed that all nine conserved residues forming the carbohydrate-binding site in most mammalian galectins are present in the putative binding site of Drgal1-L2, and that all side chains of these residues were within 0.5 Å of the equivalent side chains of the bovine spleen galectin (Figure 6A). Drgal1-L2 also has a histidine at position 52, like mammalian and toad galectin-1, and chicken-16 galectin, whereas the congerins, skin mucus galectins from the conger eel (*C. myriaster)* [84,85] have a glycine in the same position. The crystal structure of congerin II suggests that the tyrosine at position 51 in congerin I and II takes the place of His 52 in protein carbohydrate interactions. The Drgal1-L2 model also reveals that in the loop between F4 and F5 from bovine spleen galectin, a tyrosine has been replaced by a serine in Drgal1-L2. Primary structure analysis reveals the presence of two additional residues at positions 66 and 67 in congerin I, and an additional residue at position 67 in congerin II, as compared to the zebrafish and bovine spleen prototype galectins. When Drgal1-L2 was modeled on the structures of congerins I and II [84,85], these differences resulted in a larger loop between S5 and S6, thus generating

**Table 2.** Current status of the characterization of the zebrafish galectin repertoire [20]

| *Subunit structure* | *Designation*[a] | *Purification* | *Carbohydrate specificity* | *Endogenous ligand* | *Clone*[b] | *Gene org/ mapping* | *Recombinant expression*[c] | *Antibodies* | *Ontogenic expression* | *gal-MO*[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| Proto | Drgal1-L2 | Y[e] | Y | Y | Y | Y/Y | Y | Y | Zygotic | Y |
| | Drgal1-L2d | N[f] | N | N | Y | Y/Y | N | N | Zygotic | N |
| | Drgal1-L1 | N | N | N | Y | N/N | N | N | Maternal | Y |
| | Drgal1-L3 | N | N | N | Y | N/N | N | N | Maternal & zygotic | Y |
| Chimera | Drgal3 | N | N | N | Y | N/N | N | N | Maternal & zygotic | Y |
| Tandem-repeat | Drgal9-L1 | N | N | N | Y | N/N | Y | N | Maternal & zygotic | Y |
| | Drgal9-L2 | N | N | N | N | N/N | N | N | N | N |

[a]Based on reference 9; For example, Drgal1-L2 represents galectin-1-like lectin 2 from *D. rerio*.
[b]Drgal11-L2 was cloned from peptide sequencing of the purified protein. The clone for the isoform of Drgal1-L2 (designated Drgal1-L2d) was obtained during cloning of the Drgal1-L2 cDNA. The rest galectins were cloned from public data base (either from complete or incomplete sequences).
[c]Most galectins were cloned into the expression vector pET-30 (Novagen, Madison, WI).
[d]gal-MO represents morpholino modified antisense oligos.
[e]Y, Yes.
[f]N, not done or not determined.

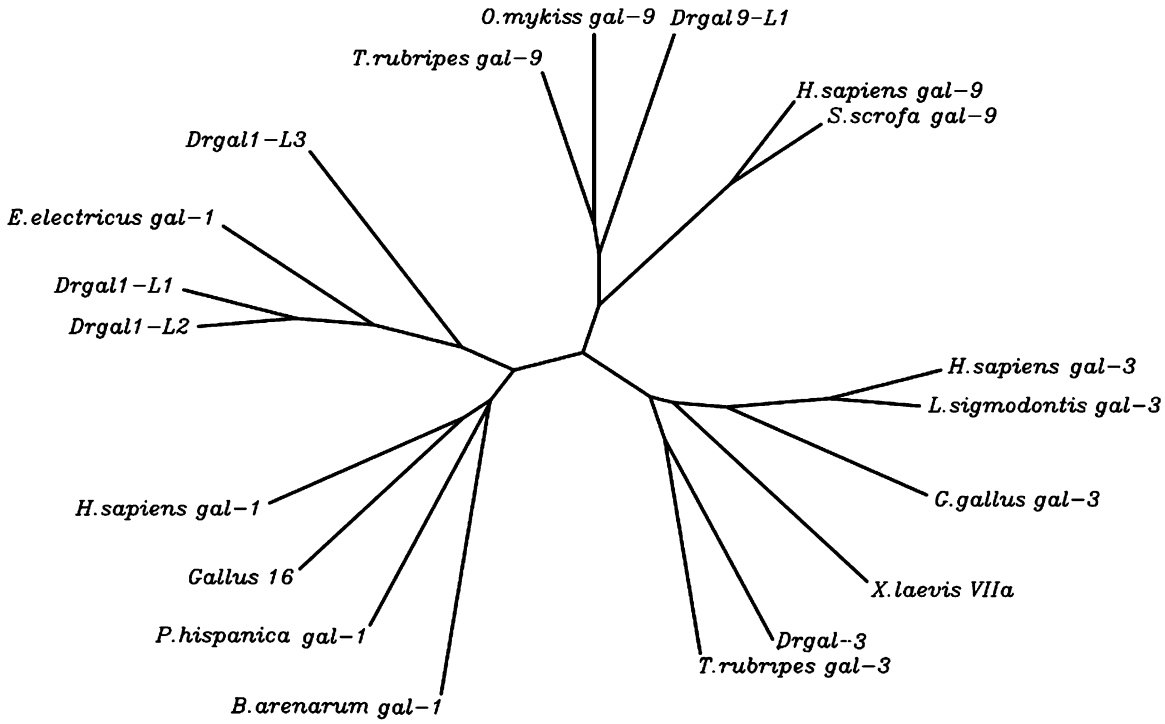


**Figure 5.** Relationship of *D. rerio* galectins to galectin family. Galectins of *D. rerio* are phylogenetically related to galectins of diverse species. The unrooted dendrogram shows the relationships of *D. rerio* prototype, chimera, and tandem-repeat galectins to these same structural types in other organisms. Following organism's name, gal-1-galectin-1 (prototype); gal-3-galectin-3 (chimera); gal-9-galectin-9 (tandem-repeat). This was generated using the computer program ClustalW (clustalw.genome.ad.jp/).

a backbone discrepancy between the congerins structure and the Drgal1-L2 model (Figure 6B). The only other backbone difference is found where congerin I has the conformationally extended N- and C-termini, where it is believed that congerin I undergoes inter-subunit strand swapping when dimerized. With respect to energy minimization, Drgal1-L2 appears to favor the bovine spleen galectin and congerin II structure at the N- and C-termini. Congerin II may have an extended binding site beyond the non-reducing end of bound galactosides. Of the six residues suggested interacting with the extended sugar, Drgal1-L2 possesses only three identical and two similar with congerin II (Figure 3). Drgal1-L2 has even fewer of these six residues in common with bovine spleen galectin. This analysis suggests that binding sites of the prototype zebrafish galectins are structurally similar to the mammalian homologues. However, they appear as less similar to the galectins from the conger eel, which are clearly non-orthologous gene products.

### *Temporal and spatial expression*

The ontogenic expression of zebrafish galectins is interesting. Drgal1-L2 is expressed post bud stage, and its expression is strikingly specific to the notochord (Figure 7) [20]. In contrast, Drgal1-L1 is expressed maternally in the oocytes. Drgal1-L3, Drgal3, and Drgal9-L1 are expressed both maternally and zygotically, ubiquitously in the adult tissues [20]. The distinct temporal and spatial patterns of expression of members of the zebrafish galectin repertoire suggest that each may play distinct biological roles during early embryogenesis.

The notochord, an early embryonic structure derived from cells in the embryonic shield [108], provides both mechanical and signaling functions. Recent studies have shown that two zebrafish notochord mutants, *grumpy* and *sleepy*, encode laminin $\beta 1$ and $\gamma 1$ [109]. This finding is consistent with the idea that galectin may be involved in notochord formation because laminins are galectin receptors. The notochord plays a very important role in the specification and differentiation of skeletal muscles. Studies by others and us have demonstrated that Hedgehog signals produced by notochord are required for induction of somitic mesoderm to form slow muscles [110,111]. Thus, disruption of notochord formation will likely result in development defects in skeletal muscle formation. Like Hedghog, Drgal1-L2 may influence notochord formation and consequently the expression of Hedghog signals and thus Drgal1-L2 may indirectly be involved in muscle cell differentiation.

## Current approaches to address biological roles of galectins in zebrafish

Several unique approaches have been established in the zebrafish model to address biological roles of genes of interest. These approaches are currently implemented in our laboratory to examine the biological roles of galectins in zebrafish. In the

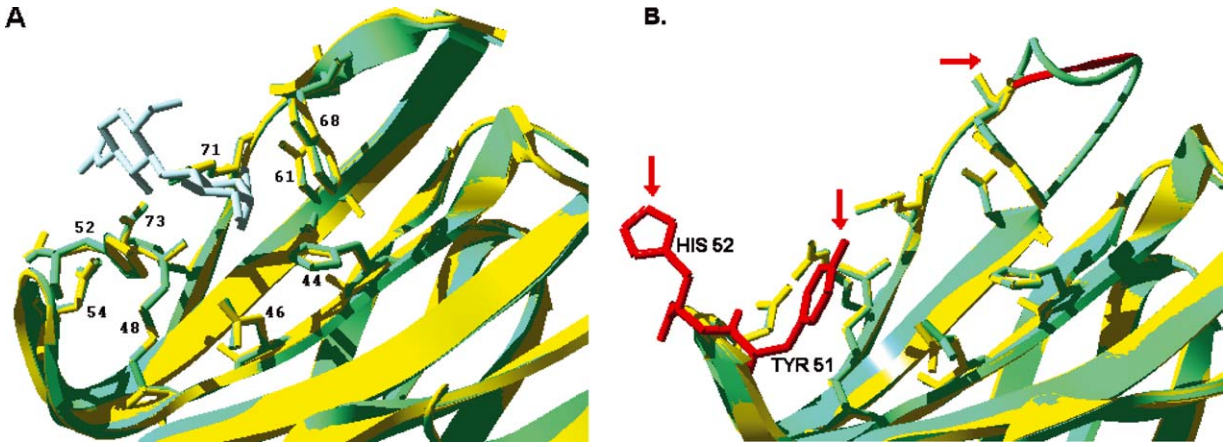


**Figure 6.** Homology modeling of Drgal1-L2. Panel A—Drgal1-L2 (yellow) was modeled to bovine spleen galectin-1 (blue), showing the sidechains of 9 conserved binding site residues of mammalian galectin-1. Panel B—Drgal1-L2 (yellow) was modeled to congerin II (blue). The same 9 sidechains are shown as previously. Major differences in the binding site architecture are the His52 of Drgal1-L2 vs Tyr51 of congerin II, and the extended loop, highlighted by the red arrow, where the model is not supported due to additional residue (Ser62) in congerin II.

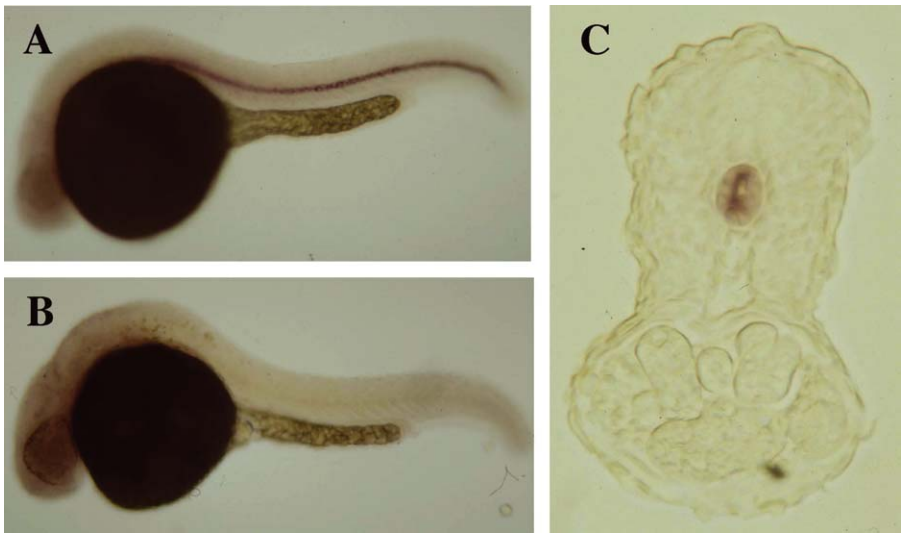


**Figure 7.** Whole mount *in situ* hybridization. A, C. *In situ* hybridization of whole zebrafish embryo (24 h post fertilization) showing Drgal1-L2 expression in notochord. A. Lateral view; B. Whole embryo (24 h post fertilization) with sense probe (-) control; C. Cross-section of trunk after whole mount *in situ* hybridization. Results are based on studies published elsewhere [20].

first approach, the expression of the selected gene is blocked by injecting a morpholino-modified antisense oligo against the corresponding mRNA that blocks protein translation [112,113] or a splicing blocker that interferes with intron/exon splicing [114]. In the second approach, ectopic expression is used to manipulate the activity of a gene product in zebrafish embryos by either mRNA injection or DNA injection [111]. The expression and activity of the gene product can be inhibited or increased by specifically expressing a dominant negative or a full-length active form of that protein, respectively. The expression can be targeted to the specific tissue/organ of interest by using tissue-specific promoters [111]. In the third approach, mutants that affect the expression of the gene of interest are analyzed to determine possible interactions between the mutant and the gene of interest, and the potential functions of gene of interest. The last category includes a series of approaches such as bacterial challenges through incubation or injection into zebrafish embryos that pertain to elucidation of the potential role(s) of the proteins of interest in innate immunity during embryogenesis [115].

### *Antisense morpholino knockdowns*

To examine the possible function(s) of galectins in zebrafish embryos, blocking of galectins expression is performed by

injection of morpholino-modified antisense oligo targeted to the galectin 5'UTR sequence near the ATG start site. To determine if the injection of antisense oligos effectively block galectin expression, whole mount antibody staining is performed on injected and uninjected embryos and compared the results. Histological analysis is performed with injected embryos to determine any developmental defect. Co-injections are also performed with the galectin mRNAs to rescue the developmental defect. Co-injection should rescue the development defect if the phenotype is galectin-specific.

### *Expression of ectopic galectin mRNA*

*Overexpression of the gene of interest.* The phenotypic consequences of overexpression of the gene of interest can provide substantial information about its biological role [111,116]. For this purpose, the cDNA is cloned into a suitable vector and capped mRNAs are transcribed *in vitro* from the linearized plasmid DNA. The capped mRNAs are injected into 1–4 cell stage zebrafish embryos through microinjection. The injected mRNAs are distributed globally in the embryos. Consequently, proteins are made in ectopic sites that normally do not express these proteins. For example, injection of mRNAs encoding Drgal1-L2 into zebrafish embryos results in ectopic expression in many cell types in addition to notochord cells that express the endogenous Drgal1-L2. Thus, this approach provides a useful tool to analyze the biological role of Drgal1-L2.

### *Repression of galectin function by a galectin dominant negative construct*

Another powerful approach to analyze gene function in zebrafish embryos is the use of "dominant negative" approach, which is used to specifically block the function of a gene product in a specific location. Proto galectin is a dimer of two identical non-covalently linked subunits, each of which houses carbohydrate-binding site on the opposite face [15]. Mutating the carbohydrate-binding site but keeping the dimer interface unaltered will create a mutant protein that can dimerize with the endogenous galectin, but is unable to bind to carbohydrate on the mutant subunit side. Consequently, the wild type-mutant heterodimer will fail to establish an interaction between cells or between cell and extracellular matrix. Thus, the mutant protein will act as a dominant negative that can interfere with activity of the endogenous galectin.

*Identification of notochord- and muscle-specific promoters.* Although ectopic expression through mRNA injection has been successfully used to study the function of many genes, the drawback of this approach is that the expression is not tissue-specific. As indicated above, Drgal1-L2 is expressed in the notochord of zebrafish embryos, and the ideal approach to analyze its function is to disrupt its activity specifically in the notochord. For this purpose, a promoter that targets protein expression to the notochord is identified and characterized from the *tiggywinkle hedgehog* (*twhh)* gene [117]. As notochord influences the specification and differentiation of skeletal muscles, it is of interest to specifically express wild type or mutant galectins in muscle cells. To this goal, muscle-specific promoters derived from zebrafish myoD and myogenin genes have also been identified and their activity analyzed in zebrafish embryos [118]. These promoters will be useful for expressing galectin genes in a tissue-restricted manner.

### *Analyses of notochord mutants for expression of Drgal1-L2*

The notochord specific expression of Drgal1-L2 suggests that it may play a role in notochord formation. The *in vivo* requirement of laminin $\beta 1$ and laminin $\gamma 1$, which are galectin ligands, for the differentiation of chordamesoderm to notochord [109], suggest that the mutants *grumpy* and *sleepy*, two zebrafish loci known to control notochord formation and encode laminin $\beta 1$ and laminin $\gamma 1$, respectively, should be examined in detail with regard to galectin function. Several zebrafish mutants with defects in notochord development have been generated [119,120]. These mutants have been classified into early or late defects. The early defect mutants include *ntl, flh, doc* and *mom,* and are involved in initial specification and differentiation of notochord cells. Later defect mutants, such as *sly, gup, qam, sno, drb, git, bal, blo, pun* and *kon* are primarily involved in maturation of the notochord. Although initial notochord formation appears normal in these mutants, the notochord cells failed to vacuolate. Except for *sly* and *qam* which have been cloned and encode laminin $\beta 1$ and laminin $\gamma 1$, respectively, most of the mutants have not been mapped. It is interesting to note that some of the mutants, such as *kon*, showed both notochord and somite defect, and reduced mobility [119].

### *Role(s) of galectins in innate immunity*

Several approaches are currently taken to study the function(s) of galectins in zebrafish upon immune challenge. These include: (1) challenge wild type embryos, hatchlings (before and after 4 days) and adults with LPS, heat-killed and live bacteria of the three types (with PBS injections and non-injected specimens as controls) in a dose-response format; (2) challenge embryo knockdowns for each galectin and compare with the wild type; (3) challenge adult transgenic zebrafish expressing dominant negative for each galectin and compare with the wild type. For each group, experiments are performed to assess (a) Quali- and quantitative changes on the patterns of temporal expression of galectins by standard and real-time RT-PCR; (b) Quali- and quantitative changes on the patterns of spatial expression (organ/tissue distribution) of galectins by *in situ* hybridization and immunostaining; (c) Quali- and quantitative aspects of the local acute inflammatory response at the injection site by histological analysis (magnitude of the cellular response), composition of the cellular response (cell types) by using cell-specific markers for in situ hybridization and immunostaining; (d) Quali- and quantitative aspects of the systemic humoral acute inflammatory response, focusing on interferon expression

by RT-PCR; and (e) Developmental course of the embryos, hatching rates, hatchling and adult survival rates.

## Conclusions

Because of its external fertilization, rapidly developing transparent embryos, variety of established techniques for manipulation of gene expression, and a growing collection of mutations affecting early embryonic development that have been characterized and mapped, zebrafish (*D. rerio*) exhibits substantial experimental advantages over murine models for developmental studies. Zebrafish is endowed of a galectin repertoire that includes members of the three galectin subtypes, proto, chimera, and tandem-repeat, although less diversified relative to that of mammals. Further, structural analysis of selected zebrafish galectins suggests that their binding properties are very similar to the mammalian equivalents. Members of the zebrafish galectin repertoire exhibit unique temporal and spatial gene expression during early development. In this context, we propose the use of this species as a model organism for the elucidation and characterization of the biological role(s) of galectins in vertebrates, including mammals.

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