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Insect galectins: Roles in immunity and development

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As evidenced by the reviews in this special issue of *Glycoconjugate Journal*, much research is focused on determining functions for mammalian galectins. However, the identification of precise functions for mammalian galectins may be complicated by redundancy in tissue expression and in target cell recognition of the many mammalian galectins. Therefore, lower organisms may be useful in deciphering precise functions for galectins. Unfortunately, some genetically manipulable model systems such as *Caenorhabditis elegans* may have more galectins than mammals.

Recently, galectins were identified in two well-studied insect systems, *Drosophila melanogaster* and *Anopheles gambiae*. In addition to the powerful genetic manipulation available in these insect models, there is a sophisticated understanding of many biological processes in these organisms that can be directly compared and applied to mammalian systems. Understanding the roles of galectins in insects may provide insight into precise functions of galectins in mammals. *Published in 2004.*

Keywords: **galectin, innate immunity, development, *Drosophila melanogaster*, *Anopheles gambiae***

Abbreviations: **Dmgal, *Drosophila melanogaster* galectin; CRD, carbohydrate recognition domain; EST, expressed sequence tag; MBP, mannose binding protein; LPS, lipopolysaccharide.**

Introduction

The galectins are a phylogenetically ancient lectin family that are expressed in species that diverged up to 800 million years ago [1,2]. In mammals, 14 galectins have been identified [3]. Galectins have also been found in non-mammalian organisms such as sponges, worms, reptiles, fungi, eels, and fish. In addition, putative galectins are present in the genome of viruses, plants, and insects [1]. In many cases multiple galectins are expressed within a single organism. For example, in *C. elegans*, 2 galectins have been characterized and a GenBank^{TM}screen identified 26 putative galectins [1].

The evolutionary conservation of galectins and their multiplicity within a single organism imply important biologic functions for galectins. In mammals, galectins influence cell adhesion, proliferation, migration, apoptosis, inflammation, and immunity [3–6]. In addition, exogenous administration of galectin-1 was therapeutic in animal models of autoimmune disease such as arthritis, multiple sclerosis, and myasthenia gravis, suggesting an immunoregulatory role for galectins [3,5]. However, functions for galectins in lower organisms remain largely unexplored and raises the question, what common functions may galectins have in disparate organisms such as insects and mammals?

Like mammals, insects have a complex and effective innate immune system that provides powerful resistance against microbial infections [7–10]. The microbial antigens recognized by the innate immune system typically consist of repeating saccharide units found on microbial surface glycoproteins, such as lipopolysaccharide (LPS) on Gram negative bacteria, peptidoglycan on Gram positive bacteria, and high mannose glycans on yeast [7–10]. These repeating saccharide units are recognized by pattern recognition receptors (PRRs), such as Toll and mannose binding protein (MBP) [8,10]. Recognition of microbial saccharides by PRRs in insects results in the release of anti-microbial peptides such as defensins, drosomycins, and cecropins that directly kill the pathogens [8]. Alternatively, pathogens may also be phagocytosed by insect hemocytes following the recognition of microbial antigens [8]. In insects, innate immunity is the only anti-microbial defense mechanism. This evolutionarily ancient defense mechanism is also present in mammals, but in mammals, innate immunity provides the first line of defense against microorganisms and triggers the more sophisticated adaptive immune system.

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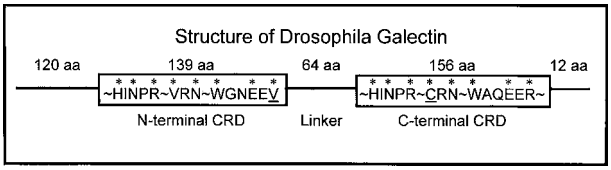

Figure 1. Schematic of the structure of Dmgal. Asterisk denotes conserved amino acids involved in saccharide binding. Amino acid substitutions are underlined. Reproduced from *The Journal of Biological Chemistry*, **277**, 13091–8 (2002) by copyright permission of The American Society for Biochemistry and Molecular Biology, Inc.

Just as insects and mammals share PRRs, such as Toll and MBP, that activate the innate immune system, they may also share effector mechanisms that assist in clearing microbial infections. Galectins have been shown to be effector molecules in the humoral innate immune response [11]. In sponges and mammals, galectins compose an alternate complement activation system along with MBP and complement that leads to the opsinization and engulfment of microbes or the formation of pores in the microbial membrane causing cell lysis [11]. Furthermore, galectin-1 was shown to inhibit the replication of *Trypanosoma cruzi* within infected murine macrophages and led to apoptosis of the infected macrophages [12]. Galectins may function similarly in the insect immune system. Kafatos *et al.* identified a putative galectin homologue that was up regulated in *Anopheles* mosquitoes during the mosquito immune response to infection with bacteria and malarial parasites [13–15]. This putative *Anopheles* galectin was proposed to be involved in mosquito innate immunity.

Galectins from insects

Anopheles gambiae and *Drosophila melanogaster* are well-studied insect models that may be useful for elucidating precise functions for galectin family members. We recently identified a *Drosophila* galectin homologue (Dmgal, GenBank™ accession number AF338142) by using 5' and 3' RACE and gene specific primers directed against an expressed sequence tag (EST, LP06039.5prime) with sequence similarity to the carbohydrate recognition domain of galectin family members [16]. Figure 1 shows the structural organization of Dmgal that was derived following the comparison of the deduced amino acid sequence (Figure 1) of Dmgal with known mammalian galectin sequences and structures. Dmgal is a 58 kDa protein composed of 2 carbohydrate recognition domains (CRDs) connected by a peptide link; this structural organization classifies Dmgal in the group of tandem repeat type galectins. In contrast, the putative *Anopheles* galectin is predicted to contain only one CRD; this suggests that the putative *Anopheles* galectin is a member of the prototype group of galectins.

The deduced amino acid sequence of Dmgal shares additional features with galectin family members [17,18]. Dmgal did not contain a putative transmembrane domain or Ca^{+2} binding domain, consistent with the soluble nature of galectins and their Ca^{+2}independent binding [17,18]. In addition, Dmgal did not contain a classical secretion signal peptide, suggesting that, like mammalian galectins, Dmgal is secreted by a non-classical secretion pathway [19]. In contrast to mammalian galectins, Dmgal contains a relatively long 120 amino acid N-terminal sequence before the first CRD. The function of this sequence is not known.

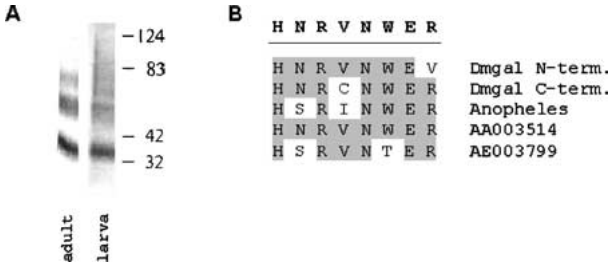

Figure 2. Additional *Drosophila* galectin homologues may exist. A. A Western blot of β-lactose binding proteins from adult and 3rd instar larval *Drosophila* isolated using β-lactose affinity chromatography and probed with anti-galectin-1 antibody. In addition to the 58 kDa Dmgal, two proteins with a M_r of 38 kDa and 72 kDa were enriched in the column eluate and cross-reacted with anti-human galectin-1 antibody. B. The consensus motif of conserved amino acids involved in galectin-1 carbohydrate recognition (top) compared to the corresponding positions in the Dmgal, *Anopheles*, and putative *Drosophila* galectins. A BLAST search revealed 2 sequences in the *Drosophila* genome (GenBank™ accession numbers AA003514 and AE003799) containing the conserved amino acid motif involved in β-galactoside sugar recognition. Amino acids identical to the conserved motif are shaded. The position of the residues in the sequence are indicated by asterisks in Figure 3.

Since most organisms express more than one galectin [1], we looked for additional galectin homologues in *Drosophila*. β-galactoside binding lectins from adult and 3rd instar larval *Drosophila* were purified using β-lactose affinity chromatography. Immunoblotting with a rabbit polyclonal antibody raised to human galectin-1 revealed two additional proteins of 38 and 72 kDa that bound β-lactose and cross-reacted with an anti-human galectin-1 antibody [16] (Figure 2A). These proteins may represent modified or degraded Dmgal, or they may be additional members of a family of *Drosophila* galectins.

As a complementary approach to look for additional putative galectins in the *Drosophila* genome, a BLAST search of the Berkeley *Drosophila* genome project was performed with the deduced amino acid sequence of Dmgal [16]. Interestingly, one sequence contained the entire conserved motif of amino acids involved in carbohydrate recognition [20] (GenBank™ accession number AE003514), while another contained many of the conserved amino acids required for carbohydrate recognition (GenBank™ accession number AA003799) (Figure 2B). These two sequences may encode additional *Drosophila* galectins. However, more studies are necessary to determine whether the proteins are capable of binding

β-galactoside sugars: in particular, the substitution of threonine for tryptophan in sequence AE003799 would likely destroy galactoside binding, so this sequence may not encode a true galectin.

Is Dmgal a galectin?

The defining feature of galectin family members is the ability to bind β-galactoside sugars [2,17,18]. While the amino acid sequence of the Dmgal CRDs suggested that Dmgal could bind β-galactoside sugars, expression of appropriate carbohydrate ligands for Dmgal in *Drosophila* would be critical for Dmgal to exert a function *in vivo*. Although relatively little is known about glycosylation in *Drosophila*, lectin staining of *Drosophila* embryos suggested that the canonical galectin ligand, *N*-acetyllactosamine (Galβ1,4GlcNAc), is expressed during embryogenesis [21]. In addition, BLAST searches of the complete *Drosophila* genome revealed ESTs encoding the complement of glycosyltransferases sufficient to produce both N-linked *N*-acetyllactosamine and poly-*N*-acetyllactosamine glycans [22]. This suggested that *N*-acetyllactosamine-containing ligands are present in *Drosophila* and that Dmgal may bind *N*-acetyllactosamine-containing ligands *in vivo*. *In vitro*, both native and recombinant Dmgal bound to a β-lactose affinity column, but not a fucose affinity column, confirming that Dmgal recognizes β-galactoside sugars [16]. The CRD of the putative *Anopheles* galectin also contained many of the conserved amino acids involved in carbohydrate recognition; however, neither the lectin activity nor the sugar specificity of the putative mosquito galectin has been examined. Rigorous characterization of the carbohydrate binding specificity of both Dmgal and the putative *Anopheles* galectin will reveal more about carbohydrate ligands and glycosylation in these insects.

Sequence comparisons of insect galectins and selected mammalian galectins

Figure 3 shows the alignment of Dmgal and the putative *Anopheles* galectin with galectins from selected species. The deduced amino acid sequence of the putative *Anopheles* galectin shared 32% identity and 49% positivity with CRDI of Dmgal. Dmgal had a great deal of sequence similarity to the tandem repeat galectins, human galectin-4 and murine galectin-9 (30 and 28% identity, and 39 and 44% positive, respectively), while the putative *Anopheles* galectin was most similar to the N-terminal CRDs of human galectin-8 and human galectin-4 (30 and 33% identity, and 48 and 47% positive, respectively). Interestingly, the sequence similarity of Dmgal with mammalian tandem repeat type galectins, such as galectin-4, -8, and -9 was slightly greater than its similarity to the *C. elegans* tandem repeat galectin, demonstrating strong conservation across vertebrate and invertebrate species.

Further examination of the two CRDs of Dmgal demonstrated that each CRD contained many of the conserved amino acids that are required to bind β-galactoside sugars, with some substitutions as shown in Figure 2B and Figure 3 by asterisks [18,20]. In CRDI there was an Arg to Val substitution at amino acid 206. The Arg was shown to stabilize galectin-carbohydrate interactions in most galectin family members. However, in other galectins this Arg was substituted with Val (galectin-11), Lys (galectin-4 and -14, *Xenopus laevis* galectin), Ile (galectin-8), Glu (galectin-6 and -10), Thr (galectin-13), and His (*Geodia cydonium* galectin I) [23–29]. In CRD II there was a Val to Cys substitution at amino acid 406. This amino acid was also substituted with Gln and Ile in *Conger myriaster* Lec 1 and *Caenorhabditis elegans* 32 kDa galectin, respectively [30,31]. The amino acid variation between the two CRDs of Dmgal suggests that each CRD may differ slightly in its ability to bind sugar ligands or in its sugar specificity, as has been shown for human galectin-12 [32–34].

The putative *Anopheles* galectin also had some amino acid substitutions within its CRD compared to the canonical motif of amino acids involved in sugar recognition. As shown in Figure 2, Asn was substituted with Ser at amino acid 59, and Val with Ile at amino acid 68. Both of these conservative substitutions may not affect carbohydrate binding activity, although, as mentioned above, further studies are required to determine whether the *Anopheles* galectin is capable of binding β-galactoside sugars.

In summary, the amino acid sequence similarity between insect and mammalian galectins suggests that like mammalian galectins, insect galectins may cross-link glycoconjugates on opposing cell surfaces to mediate cell-cell interactions or cross-link glycoprotein receptors on the cell surface to trigger signal transduction pathways [3,4,35].

Functions for insect galectins—Immunity and development

Innate immunity

The innate immune response to pathogens consists of both humoral and cellular reactions [7–10]. In insects, the humoral reaction involves the rapid induction of proteolytic cascades culminating in synthesis of anti-microbial peptides and localized melanization and hemolymph clotting at the injury site or around invading microbes [8,9]. Insect anti-microbial peptides, such as the anti-bacterial cecropins and the anti-fungal drosomycin destroy invading microbes by disrupting the microbial cell membranes leading to cell lysis [8,9]. In insects, anti-microbial peptides are mainly synthesized by the fat body, but epithelial cells, hemocytes, salivary glands, gut tissues, and the reproductive tract can also produce anti-microbial peptides in a localized immune response [8,9].

The insect cellular reaction consists of phagocytosis or encapsulation of invading microbes by hemocytes, the circulating cells of the innate immune system [36]. Three types of hemocytes have been described; the plasmatocytes that are phagocytic and produce anti-microbial peptides, the lamellocytes that aggregate to surround and engulf large intruders,

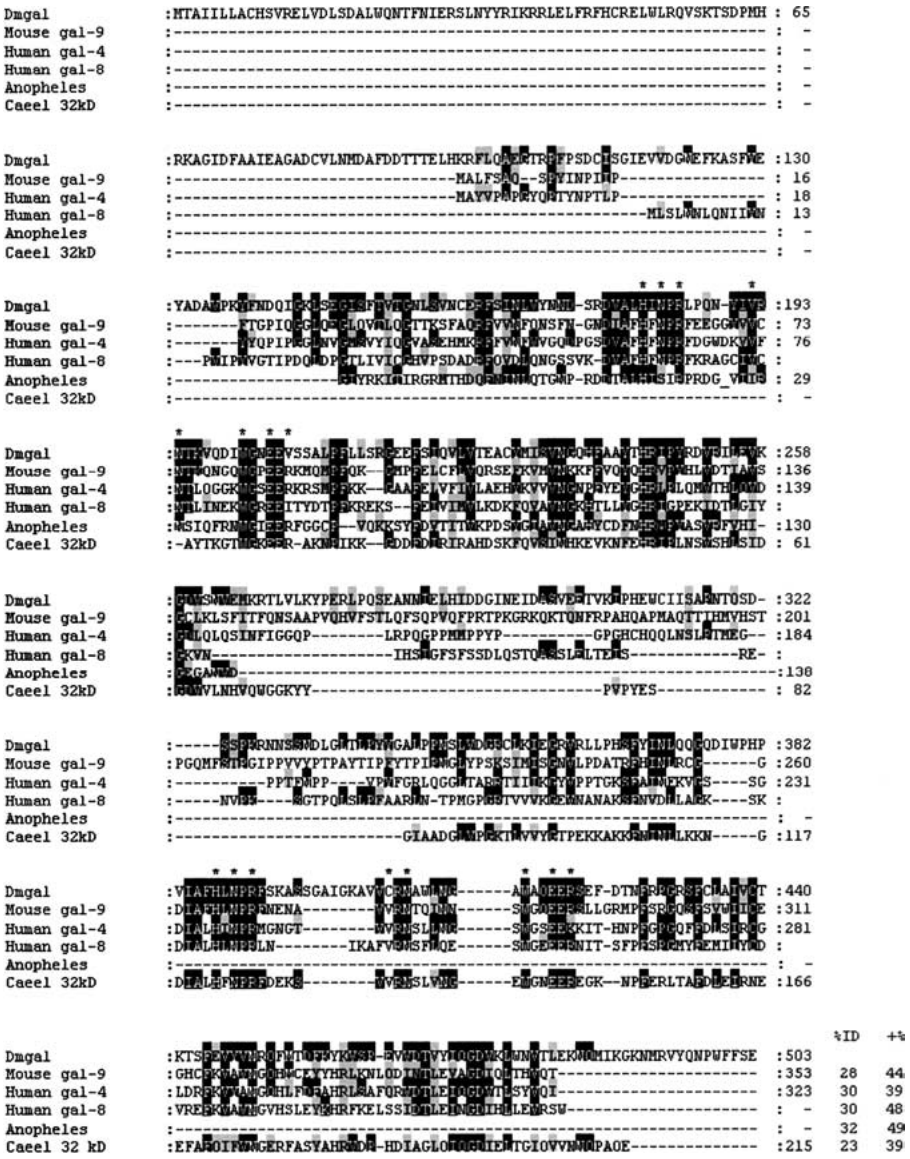

Figure 3. Amino acid alignment of insect galectins and selected mammalian galectins. The sequence alignment was produced using ClustalX (version 1.8). Similarity groups (gray shading) and identities (black shading) were generated using Genedoc (version 2.6.001) and the Blosum 62 scoring table. Asterisks denote amino acids critical for saccharide binding. Percent identities and percent positives are shown to the right. Dmgal, *Drosophila melanogaster* galectin; mouse gal-9, *Mus musculus* galectin-9; human gal-4, *Homo sapians* galectin-4; human gal-8, *Homo sapians* galectin-4; Anopheles, *Anopheles gambiae* putative galectin; Caeel 32 kD, *Caenorhabditis elegans* 32 kDa galectin.

and crystal cells that produce elements required for defense-related melanization [36].

As described above, Kafatos *et al*. demonstrated that a putative galectin homologue was up-regulated in the salivary glands and gut of *Anopheles* mosquitoes that were infected with malaria or bacteria [13–15]. During the innate immune response to these pathogens, the putative *Anopheles* galectin may function as a PRR by binding saccharide ligands on the microbial surface to trigger a host immune response [15]. Alternatively, the *Anopheles* galectin may agglutinate and opsonize bacteria and contribute to limiting bacterial growth in the midgut following blood-feeding [15].

In uninfected larval *Drosophila*, Dmgal was expressed in hemocytes (Figure 4), but not by the fat body or larval lymph glands [16]. All of the hemocytes examined expressed Dmgal, although two distinct labeling patterns were observed. In about 75% of hemocytes, galectin was localized in a large patch located at one pole of the cell, while in the remaining hemocytes, galectin was concentrated in small patches within the cytosol. These staining patterns may represent the localization of

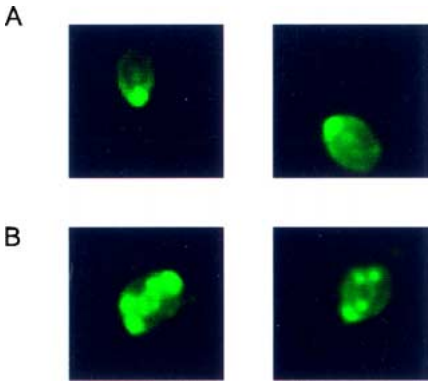

Figure 4. Dmgal is expressed in larval hemocytes. Smears of circulating hemocytes from 3rd instar hemolymph were labeled with rabbit anti-human galectin-1 antibody followed by fluorescein conjugated anti-rabbit antibody. A. Dmgal (green) was localized in a large patch at one pole of the cell in the majority of hemocytes. One 0.5 μm slice each of 2 different cells is shown. B. In the remaining hemocytes, Dmgal was localized in multiple small concentrations within the cytosol. One 0.5 μm slice each of 2 different cells is shown. The images were collected using the $\times$100 objective on an Olympus Flowview Confocal Microscope and analyzed with Fluoview Image Analysis software (version 2.1.39).

Dmgal within different hemocyte subtypes, or the localization of modified Dmgal or additional *Drosophila* galectin family members within the cytosol. Alternatively, the staining pattern may represent the unique synthesis and secretion pattern described for mammalian galectins [19,37], characterized by synthesis of galectins within the cytosol, formation of intracellular concentrations of galectin, and evagination and release of galectin-containing vesicles from the cell [19,37].

What are the possible functions for Dmgal in the fly immune system? Like mammalian galectin-1 and galectin-3, Dmgal may participate in the innate immune system of the fly by facilitating microbial recognition and/or phagocytosis [38–41]. Since Dmgal is divalent, it may also participate in hemocyte aggregation and subsequent encapsulation of microbes during infection. Alternatively, Dmgal may be released from hemocytes following an immune challenge, similar to the release of galectin-10 from mammalian eosinophils following stimulation [42], or the release of galectin-3 from dendritic cell exosomes during antigen presentation [43].

Dmgal and development

Cell surface carbohydrates and lectins play key roles in signaling, pathfinding, and the establishment of spatial boundaries and patterns during development [44,45]. Two C-type lectins, gliolectin [46] and a selectin homologue [47] function during *Drosophila* development. Gliolectin facilitated the formation of axon and glial contacts and ensured that neural and glial membranes remained in contact long enough to communicate and integrate sorting information during neural development [46] The selectin homologue was critical for proper eye and mechanosensory bristle development [47].

As mammalian galectins participate in organ development, Dmgal may likewise have a role in *Drosophila* development. Figure 5 shows the expression pattern of Dmgal during the development of wild type *Drosophila* embryos. During gastrulation, an early stage of embryogenesis that involves widespread cell migration and rearrangement, Dmgal was expressed within the invaginating furrow that forms the mesodermal layer. As embryogenesis continued, Dmgal became strongly expressed in the mesodermal and neural layers and in the invaginating foregut and hindgut. The mesoderm forms somatic and visceral muscle, endothelium, and connective tissue; these are also tissues that express galectins in mammals [6,48]. In particular, the mesoderm forms components of the *Drosophila* innate immune system, such as the fat body, lymph glands, and hemocytes.

In late stages of embryogenesis, Dmgal expression was concentrated in somatic and visceral musculature and in the central nervous system [16]. Expression of Dmgal within the musculature suggested that Dmgal may mediate the cell-cell and cell-matrix interactions required for muscle development. During muscle development in chicks, galectin is expressed at stages of myotome segregation and muscle differentiation and was proposed to modulate adhesion to the ECM during myoblast fusion [37,49]. Recently, galectin-1 was shown to convert non-myogenic dermal fibroblast to a myogenic lineage and affect terminal differentiation of myogenic cells [50]. Dmgal may have a similar effect during *Drosophila* muscle development.

Dmgal expression was also enriched in the CNS [16]. During *Drosophila* embryogenesis galactoside-containing saccharides are present in the developing tracts of the ventral nerve cord and on axons and glia that ensheath neurons and appose the axon tracts [21]. Dmgal could be a receptor for these glycoconjugates and facilitate axon guidance or fasciculation. Laminin, a known glycoprotein receptor for galectins, surrounds developing glia and axons in the *Drosophila* nervous system [51] and may be an extracellular matrix protein that binds Dmgal during neural development. In mice, galectin-1 and galectin-3 are transiently expressed by a subset of murine dorsal root ganglia and have been proposed to mediate interactions necessary for axonal fasciculation [52,53]. Furthermore, galectin-$1^{-/-}$ mice display intriguing deficits in olfactory axon pathfinding during neural development [53]. Similarly, Dmgal may mediate cell-cell interactions and migration during *Drosophila* neural development.

In *Drosophila* and mammals, the Notch receptor is important for differentiation and development in a variety of tissues, including neural [54] and immune tissues [55,56]. Recently, Stanley *et al.* found that the *N*-acetyllactosamine sequence (Galβ1,4GlcNAc) is required to regulate Notch signaling [57]. Since *N*-acetyllactosamine is a carbohydrate ligand recognized by galectins, Stanley's group proposed that a galectin may bind *N*-acetyllactosamine sequences on Notch and affect Notch-ligand interactions and activation [57]. This

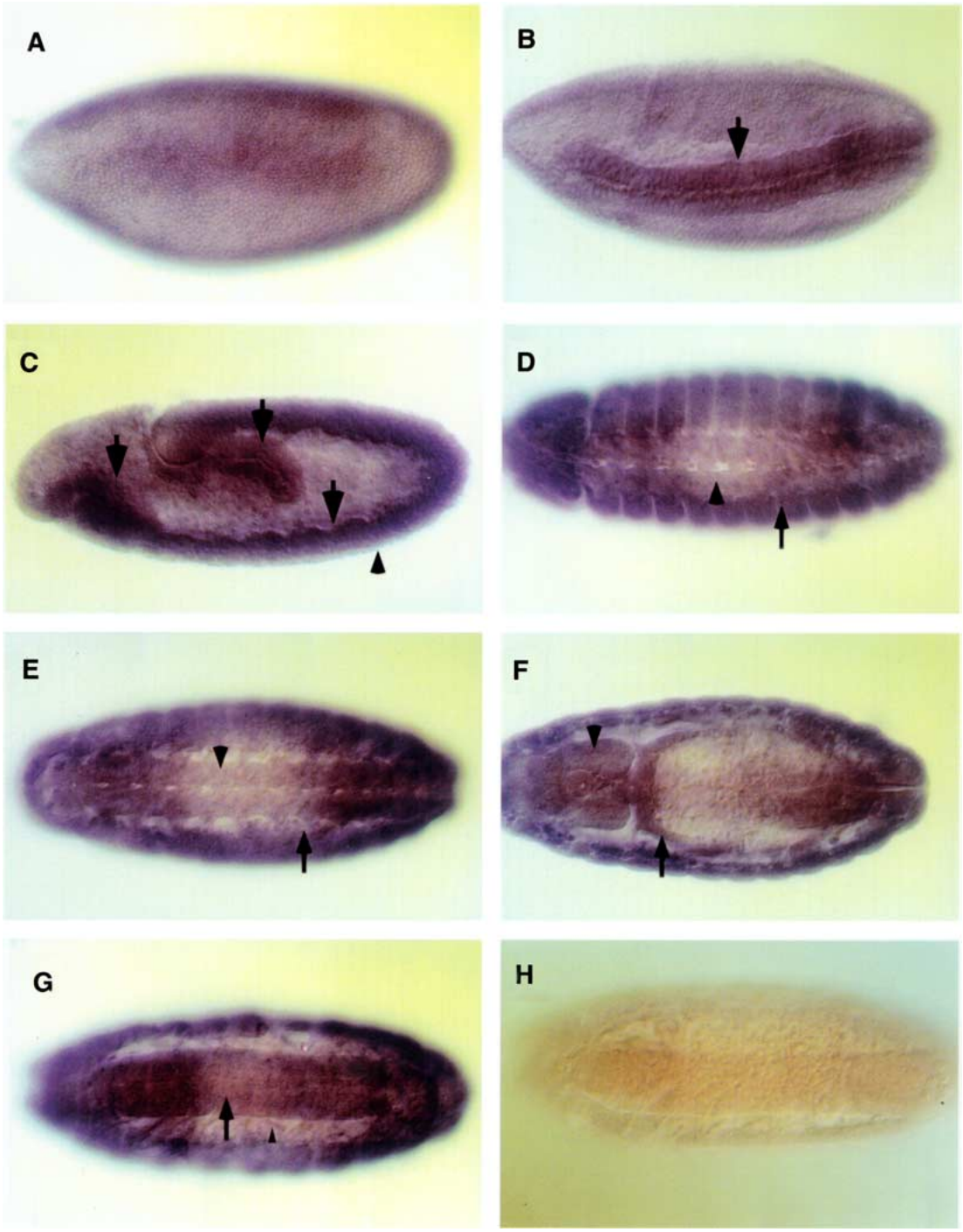

Figure 5. Dmgal expression during *Drosophila* embryogenesis. Whole mount *in situ* hybridization of Dmgal cDNA in wild type embryos. A. Dmgal mRNA is deposited maternally into the egg (lateral view, stage 5). B. During gastrulation, enriched expression of Dmgal mRNA can be detected in the presumptive mesoderm (arrow) (ventral view, stage 6). C. In the elongated germband embryo (lateral view, stage 10), Dmgal becomes strongly expressed in the neural and mesodermal layer as well as in the invaginating foregut and hindgut (arrows). In contrast, the presumptive epidermis shows only weak Dmgal expression (arrowhead). (D)–(F). In developmental stages 13–15, Dmgal mRNA becomes concentrated to the somatic and visceral musculature (arrows) and the central nervous system (arrowheads), ventral view (D) and (E); dorsal view (F). G. At the end of embryogenesis (stage 16, ventral view), Dmgal expression remains in the somatic musculature (arrowhead) and becomes strongly expressed in the central nervous system (arrow). H. Hybridization with a sense probe does not give any detectable signal (stage 16, compare with G). Reproduced from *The Journal of Biological Chemistry*, **277**, 13091–8 (2002) by copyright permission of The American Society for Biochemistry and Molecular Biology, Inc.

suggests a role for Dmgal in modulating Notch receptor signaling during *Drosophila* development.

Conclusion

The generation of genetically modified mice has revealed some exciting functions for mammalian galectins in neural development and modulation of the immune response [53,58,59]. Unfortunately, elucidation of potential *in vivo* functions for galectins by genetic manipulation in mice has been limited by the large number and potential redundancy of mammalian galectins, making the identification of galectins in other model organisms desirable. An important benefit of insect systems such as *Drosophila* is the ease of genetic manipulation and the rapid generation time. In addition, the relatively small number of putative galectins in the *Drosophila* genome [1,16] may overcome the previous problems of redundancy in other genetically manipulable models such as mice and *C. elegans*. Elucidation of the immune pathways triggered by the PRRs such as Toll has led to rapid progress in understanding innate immune functions in mammals. Likewise, understanding the roles of insect galectins in various biological processes may similarly provide insight into the functions of the many mammalian galectins.

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