



The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the *Drosophila* lymph gland



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ABSTRACT

The heparin sulfate proteoglycan Terribly Reduced Optic Lobes (Trol) is the *Drosophila melanogaster* homolog of the vertebrate protein Perlecan. Trol is expressed as part of the extracellular matrix (ECM) found in the hematopoietic organ, called the lymph gland. In the normal lymph gland, the ECM forms thin basement membranes around individual or small groups of blood progenitors. The pattern of basement membranes, reported by Trol expression, is spatio-temporally correlated to hematopoiesis. The central, medullary zone which contain undifferentiated hematopoietic progenitors has many, closely spaced membranes. Fewer basement membranes are present in the outer, cortical zone, where differentiation of blood cells takes place. Loss of *trol* causes a dramatic change of the ECM into a three-dimensional, spongy mass that fills wide spaces scattered throughout the lymph gland. At the same time proliferation is reduced, leading to a significantly smaller lymph gland. Interestingly, differentiation of blood progenitors in *trol* mutants is precocious, resulting in the break-down of the usual zonation of the lymph gland, which normally consists of an immature center (medullary zone) where cells remain undifferentiated, and an outer cortical zone, where differentiation sets in. We present evidence that the effect of Trol on blood cell differentiation is mediated by Hedgehog (Hh) signaling, which is known to be required to maintain an immature medullary zone. Overexpression of *hh* in the background of a *trol* mutation is able to rescue the premature differentiation phenotype. Our data provide novel insight into the role of the ECM component Perlecan during *Drosophila* hematopoiesis.

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Introduction

Animal cells secrete proteins that assemble into an extracellular matrix (ECM). The ECM plays important roles in determining the mechanical properties of cells and tissues, as well as in intercellular communication. ECM can also play a role in facilitating integrin mediated cell interactions (Subramanian et al., 2007). In many cells, the ECM forms a layered structure called the basal lamina or the basement membrane. In epithelia, the basement membrane assembles over the basal cell surface; in other, mesenchymal tissues, such as muscle or cartilage, basement membranes surround cells on all sides (Durbbeej, 2010). Many basement membranes are composed primarily of Laminin, type IV Collagen, Nidogen and heparin sulfate proteoglycans (HSPGs), which include Perlecan and Agrin (reviewed in Yurchenco et al., 2004; Laurila and Leivo, 1993). The heparin sulfate chains of these proteins are mainly responsible for the involvement of basement

membranes in cell–cell signaling. They bind (and thereby sequester or release in a controlled manner) ligands like Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Wnt, Hedgehog (Hh) and Transforming Growth Factor (TGF β) (Lin, 2004; Lindner et al., 2007; Yurchenco et al., 2004).

Perlecan is a large macromolecule which consists of a core protein associated with three glycosaminoglycan side chains and that occurs in basement membranes around most cells. The core protein has five distinct domains. Domains I and V, located at the N and C terminal sides, respectively, both contain binding sites for heparin sulfate side chains (reviewed in Whitelock et al., 2008). These two domains also interact with other ECM proteins, including Laminin and Collagen IV, as well cell adhesion molecules, such as integrins. Domain II is thought to interact with lipids (Fuki et al., 2000; reviewed in Whitelock et al., 2008). Domains III and IV interact with other secreted signals and ECM proteins (Friedrich et al., 1999; Iozzo, 2005; reviewed in Whitelock et al., 2008).

Given its widespread expression in basement membranes and its ability to bind to a multitude of known adhesion and signaling molecules, it is to be expected that Perlecan (along with other HSPGs) plays many different roles in tissue development and

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maintenance. Both in vitro and in vivo studies confirm this expectation. In vertebrates, Perlecan plays particularly important roles in the growth and morphogenesis of the skeleton and vascular system by modulating cell adhesion and/or the effect of the signaling molecules FGF, PDGF, and VEGF on chondrocytes, smooth muscle cells, and endothelial cells (Melrose et al., 2008; Segev et al., 2004; Whitelock et al., 2008). In *Caenorhabditis elegans*, Perlecan mediates the binding of muscle cells to epidermal cells via integrins (Rogalski et al., 2001). In *Drosophila*, the Perlecan homolog was originally identified as a mutation affecting postembryonic growth of the central nervous system. In larvae carrying this mutation, called *trol*, neuroblasts show a strongly decreased rate of proliferation (Datta, 1995). This phenotype was shown to be mediated by an effect of *trol* on FGF and Hh signaling (Caldwell and Datta, 1998; Lindner et al., 2007; Park et al., 2003). The effect of *trol* on proliferation is not confined to the CNS; experiments looking at hemocyte number in *trol* mutants have also shown a significant drop in circulating plasmatocyte numbers (Lindner et al., 2007). Further studies have also shown the expression of *trol* in the cardioblasts, visceral mesoderm and hindgut of stage 15 *Drosophila* embryos (Friedrich et al., 2000).

Comparisons of the human Perlecan gene to *trol* have found 34% sequence identity in domain III, 24% identity in domain IV and 30% identity in domain V. No significant similarity was seen in domains I or II (Murdoch et al., 1992; Park et al., 2003). In *Drosophila*, *Trol* is expressed strongly in basement membranes of embryonic tissues, where it interacts with Laminin and Collagen (Urbano et al., 2009). It also appears around hemocytes, vascular cells, and the hematopoietic organ (“lymph gland”) of the late embryo. Given the reported effect of *trol* on blood cell number, we embarked on a more detailed analysis of the role of *trol* in *Drosophila* hematopoiesis.

The blood, or hemolymph, of *Drosophila* contains three major types of blood cells (hemocytes), called plasmatocytes, crystal cells and lamellocytes. Plasmatocytes act as macrophages during development, and together with crystal cells, play a role in immunity and response to injury (Crozatier and Meister, 2007; Martinez-Agosto et al., 2007). These two cell types comprise the hemocytes most commonly seen under non-immune challenged conditions. Lamellocytes are very rare under normal conditions. In cases of immune challenge, their numbers increase and they act to neutralize objects too large to be phagocytosed.

Hemocytes are produced during two phases of development. The first phase of hematopoiesis takes place in the head mesoderm of the early embryo; hemocytes produced during this phase populate the embryo and the circulating hemolymph of the larva. The second phase of hematopoiesis takes place in the lymph gland of the larva, a solid hematopoietic organ located alongside the dorsal vessel (“heart”). The lymph gland derives from a small population of hematopoietic blood progenitors that first appear in the trunk mesoderm of the embryo, consolidate into the lymph gland, and then proliferate during the larval stage. By late larval stages, the lymph gland has grown into a series of several paired lobes which flank the dorsal vessel. Differentiation of hematopoietic progenitors into mature blood cells takes place in the periphery (cortex) of the large, anteriorly located primary lobe. A specialized subpopulation of hemocytes, called the posterior signaling center (PSC), signal to the medullary zone via the Hh pathway to maintain cells in an undifferentiated state (Mandal et al., 2007). Aside from Hh, the Wg signal (expressed in the medullary zone) and Adenosine deaminase growth factor A (Adgf-A), produced by differentiating cells in the cortical zone, antagonize prohemocyte differentiation (and prolong proliferation) in the medullary zone (Sinenko et al., 2009; Mondal et al., 2011). Differentiated hemocytes are released from the lymph gland into circulation during early metamorphosis (Lanot et al., 2001;

Grigorian et al., 2011). During this phase, the entire lymph gland dissociates; adult flies lack a solid hematopoietic organ. A lymph gland similar to that described for *Drosophila* has been documented for many insects (reviewed in Grigorian and Hartenstein, 2013). Similarities to the hematopoietic tissue of vertebrates are present; even though a prominent “stroma” (represented in the vertebrate bone marrow by the network of capillaries and reticular cells) is missing in invertebrates, cells described as “reticular cells”, surrounding prohemocytes and possibly acting as stem cells, have been described in several insect species (Hoffmann et al., 1979). In all insects investigated, profuse lamellae of ECM, formed by proteins that are found ubiquitously in basement membranes and other ECM assemblies of *Drosophila* and vertebrates (reviewed in Grigorian and Hartenstein, 2013), were observed.

In this paper, we show that Perlecan/*Trol* is expressed in basement membranes that both surround the surface of the lymph gland and that form discrete chambers within the lymph gland interior. Loss of *trol* is associated with a dramatic change in the texture of the ECM: instead of forming thin membranes around blood cells, the matrix appears as a spongy mass filling large areas of the lymph gland. Concomitantly, proliferation of blood progenitors is reduced, and the lymph gland is significantly reduced in size and cell number. Finally, the altered composition of the ECM resulting from a loss of *trol* is associated with premature hemocyte differentiation. This phenotype can be rescued by the ectopic expression of Hh. We propose that *trol* is required for the organization of the lymph gland ECM, and the proper release and/or distribution of the Hh ligand throughout the lymph gland.

Material and methods

Fly lines

*y trol*⁸ w/Bnsn (Park et al., 2001), *y trol*⁴ w/Bnsn (Park et al., 2001), *y trol*^{SD} w/Bnsn (Datta and Kankel, 1992), ZCL1973X (Kelso et al., 2004), *trol*-GFP (Lindner et al., 2007; Medioni and Noselli, 2005; Kelso et al., 2004), *domeless*-Gal4 (Jung et al., 2005; Mandal et al., 2007; Ghigliione et al., 2002), *Antennapedia*-Gal4 (Mandal et al., 2007; Emerald and Cohen, 2004), *elav*-Gal4 (Bloomington Stock Center), *Tubulin*-Gal4 (Bloomington Stock Center), *Collagen*-Gal4 (comprised of a regulatory region, located between the adjacent collagen type IV genes *Cg25C* and *Viking*, fused to a Gal4; Asha et al., 2003), UAS-*trol* RNAi (Vienna *Drosophila* RNAi Center; ID# 24549), *heatshock-hh* (*hh* fused downstream of a Hsp70 promoter; Ingham, 1993; Park et al., 2003), *hh*^{AC} (Bloomington Stock Center), *egfr*^{f2} (Bloomington Stock Center), Oregon R (Bloomington Stock Center), *Hemolectin*Δ-DsRed (Makhijani et al., 2011).

Immunohistochemistry

α-Antennapedia (Mouse; 1:4; Developmental Hybridoma Bank), α-BrdU (Rat; 1:100; Abcam), α-Cleaved Caspase 3 (Rabbit; 1:100; Cell Signaling Technology, Inc.), α-Collagen IV (Mouse; 6G7; 1:25–1:50; Murray et al., 1995), α-GFP (Mouse; 1:400; Sigma), α-GFP (Rabbit; 1:2000; Molecular Probes), α-Laminin (Rabbit; 1:500; Fessler et al., 1987), α-Odd (Rabbit; 1:500; Ward and Skeath, 2000), α-P1 (Mouse; 1:10; Kurucz et al., 2007), α-Perlecan domain V (Rabbit; 1:1000; Friedrich et al., 2000), α-Peroxidase (Mouse; 1:500; Nelson et al., 1994), α-ProPo (Rabbit; 1:1000; Muller et al., 1999). All secondary antibodies were acquired from Jackson ImmunoResearch. Both Cy3 and FITC were used at a dilution of 1:200. Cy5 was used at a dilution of 1:100. Immunohistochemistry was carried out following standard protocols (Ashburner, 1989). Some preparations were counterstained with

the nuclear marker Toto-3 iodide (Invitrogen; added at a concentration of 1:500 to the Vectashield in which the samples were mounted).

Mutant selection and larval fixation

Trol homozygous mutants were selected during 1st instar stage via yellow mouth hook color. Larvae were then allowed to mature on food plate till stated time point. All Larval lymph glands were dissected by either pulling out mouth hook with the lymph gland attached or pinning the larva dorsal side down and filleting it. Tissue fixed for 20 min in a 1:3 solution of 37% Formaldehyde: 1X PBS. Lymph glands then washed with 1X PBT (0.1%), 3 times for 15 min each at room temperature while rotating.

BrdU feeding, fixation and staining

56 h larvae were placed on a fly food plate containing 1 mg/ml of BrdU and left at 25 °C for 30 min. Larvae were then shifted to regular fly food plates and left at 25 °C until they were dissected at 73 h. Tissue was then fixed in a 1:3 solution of 37% Formaldehyde: 1X PBS for 20–30 min. Staining was carried out as described previously (Grigorian et al., 2011).

Heatshock

WT, *hs-hh*, *trol*⁸ and *hs-hh; trol*⁸ fly lines were allowed to lay eggs over the weekend on food plates. Larvae were shifted to 37 °C overnight (food plate was placed upside-down to better withstand drying). Larvae were then shifted to 29 °C for two days before being selected, dissected, fixed as described above and labeled with antibodies. All larvae dissected were 72–132 h post hatching (raised at 29 °C, except during heat shock).

Electron microscopy

Larval lymph glands were dissected and fixed as described previously (Grigorian et al., 2011) Blocks were sectioned (60 nm) and mounted on net grids (Ted Pella). Sections were treated with uranyl acetate and lead citrate. Samples were sectioned by the Microscopic Techniques Laboratory and examined using the JEOL 100CX transmission electron microscope at the Electron Microscope Laboratory of the UCLA Brain Research Institute.

Results

Trol is a component of the basement membranes that compartmentalize the lymph gland

In order to assess the expression pattern of *trol* in the heart and blood system of *Drosophila*, we utilized the GFP protein trap line ZCL1973X (Kelso et al., 2004), as well as an antibody against the Trol protein (Friedrich et al., 2000). During embryogenesis, Trol expression becomes visible around cardioblasts at stage 14 (Fig. 1A). By stage 16, Trol expression also surrounds pericardial nephrocytes and the surface of the lymph gland, a paired cluster of approximately 30 hematopoietic progenitors (Fig. 1B). Shortly after larval hatching, Trol covers each individual hematopoietic progenitor of the lymph gland. Expression is particularly strong around cells of the PSC, labeled with an antibody against Antennapedia (Antp) (Fig. 1C').

By the 2nd instar (56 h), the lymph gland has expanded, and a separate central medullary zone (hematopoietic progenitors) has become morphologically distinct from an outer, more loosely packed cortical zone, in which some cells show expression of

differentiation markers, such as Peroxidase (Pxn) (Jung et al., 2005). At the same time, secondary and tertiary lobes appear posterior to the large primary lobes. Trol expression forms a solid layer around the surface of primary and secondary lobes. Within the lobes, Trol expression is seen strongly around clusters of cells; faint Trol signal appears around individual cells. In other words, the Trol pattern outlines a system of chambers that each contain small clusters of hematopoietic progenitors or differentiated hemocytes. These chambers are smaller in the medullary zone than in the cortical zone (Fig. 1D–E), and are all but absent in the secondary/tertiary lobes (Fig. 1E). Two other major components of basement membranes, laminin and collagen IV, are co-expressed with Trol in the lymph gland, as seen via antibody staining (Fig. 1F–G'). It should be mentioned that, while it has been shown that hemocytes and their progenitors themselves express the genes encoding these ECM proteins (Knibiehler et al., 1987; Lunstrum et al., 1988; Kusche-Gullberg et al., 1992), other organs such as the fat body can also contribute to the ECM of the lymph gland, as has been demonstrated for the basement membranes of imaginal discs (Pastor-Pareja and Xu, 2011), and is further supported by data reported below.

Electron microscopically, the structures containing Trol, Laminin and Collagen IV are revealed as typical basement membranes, forming 20–40 nm thick, electron dense layers of granular material (Fig. 1H–J). Clefts of variable diameter separate basement membranes and hemocytes. Thus, in many places, basement membranes adhere closely to the surface of the hemocyte (small arrow in Fig. 1J). At other locations, basement membranes have separated from the hemocytes (large arrow in Fig. 1J). Basement membranes of the lymph gland persist for several hours after hemocytes are released during the pupal stage (Grigorian et al., 2011), attesting to the fact that these membranes are extraordinarily stable structures.

The chambered appearance of the basement membranes appears to be related to hemocyte proliferation. Thus, short pulses of BrdU fed to larvae, followed by a chase, resulted in 4–8 cell clones of labeled cells which frequently coincided with chambers (Fig. 2A–D). We propose a process where Trol (and other ECM proteins) is continuously secreted by a hematopoietic progenitor to form a basement membrane around the cell. As the hematopoietic progenitor divides, the newly formed membranes in between the daughter cells initially lack a coat of ECM, whereas this coat is already present on all other sides. In this manner, as proliferation continues, the membranes facing the “inside” of the clone are thinner (small arrow in Fig. 2D) than those facing the outside (large arrow in Fig. 2D)

Loss of trol causes a change in the ultrastructure of the lymph gland ECM

In order to assess the role of *trol* in hematopoiesis, we investigated three loss of function alleles, *trol*^{SD}, *trol*⁴ and *trol*⁸ (Lindner et al., 2007). *trol*⁸ (the allele used in the following for phenotypic analysis) showed a complete loss of Trol protein expression (Fig. 3D), whereas residual Trol signal, visible as a sheath encasing the lymph gland, remained in the other two alleles (Fig. 3B,C). To learn more about the tissue-specific requirement of *trol*, we used the UAS-Gal4 system to drive a *trol*-RNAi construct with *domeless* (*dome*)-Gal4 (medullary zone, but also nervous system; Fig. 3E), *Antennapedia* (*Antp*)-Gal4 (PSC; not shown), *Hemolectin* (*Hml*)-Gal4 (cortical zone; not shown), *Collagen* (*Cg*)-Gal4 (cortical zone, fat body; Fig. 3F), *Tubulin* (*Tub*)-Gal4 (ubiquitous; Fig. 3G), and *elav*-Gal4 (brain; Fig. 3H). Ubiquitous and medullary zone-directed expression of *trol*-RNAi

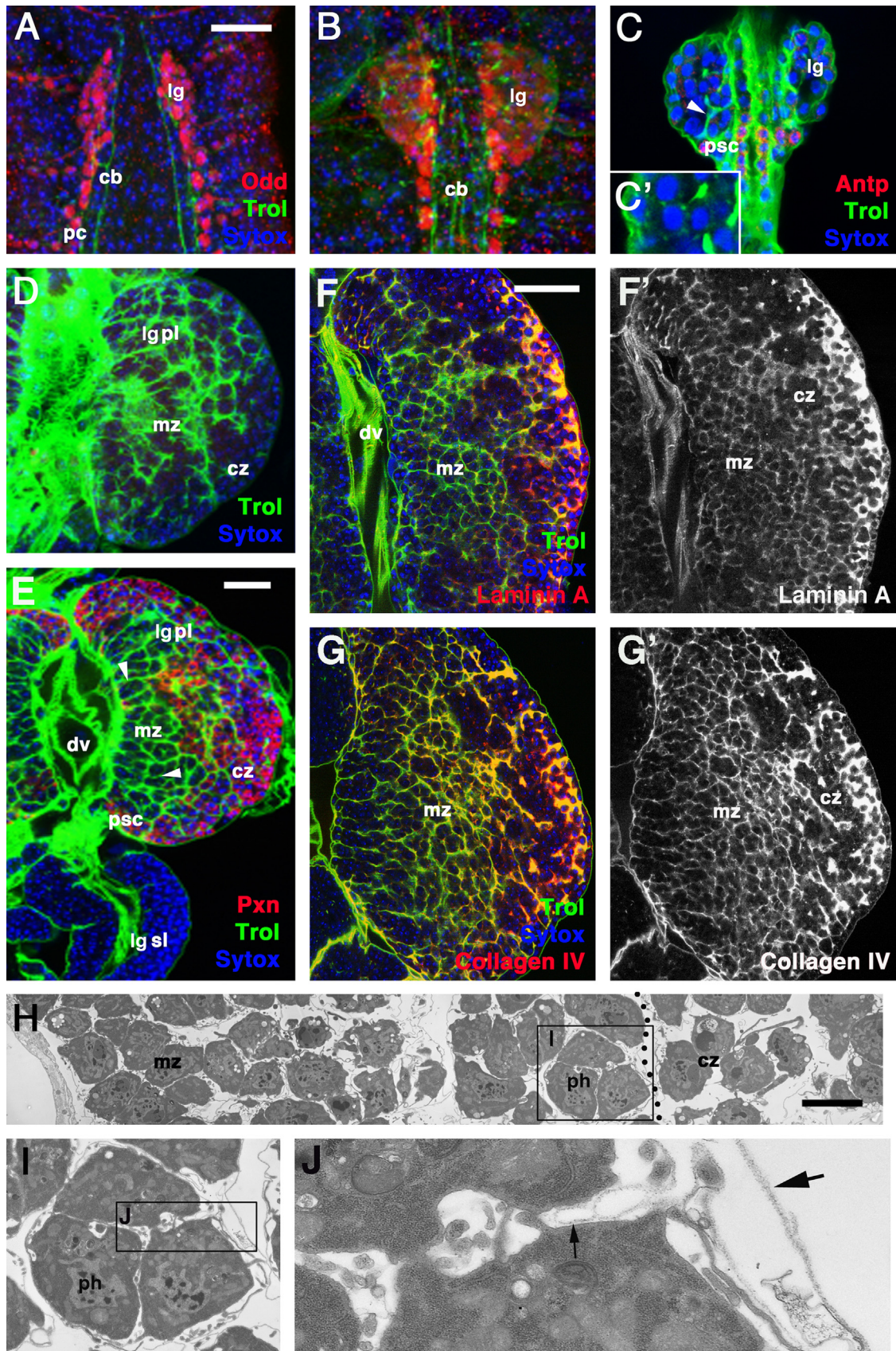


Fig. 1. Expression of Perlecan/Trol in the lymph gland and dorsal vessel. A–B: Dorsal view of stage 14 (A) and stage 16 (B) embryos. Trol expression is visualized by GFP (green) driven by the ZCL1973X line. The lymph gland (lg) and pericardial cells (pc) are also labeled with anti-Odd antibody (red). Nuclei are labeled with Sytox (blue). C: First instar larva (4–6 h post hatching). Trol expression has increased and starts to surround individual hematopoietic progenitors (arrowhead). The Posterior Signaling Center (PSC) is labeled with anti-Antennapedia (Antp; red) antibody. Nuclei are labeled with Sytox (blue). D: Second instar larva (56 h post hatching). Trol expression increases and becomes more concentrated in the center of the lymph gland, the incipient medullary zone (mz). E: Third instar larva (96 h post hatching). Trol expression is strong in the medullary zone and outlines chambers that include multiple cells (arrowhead; see also Fig. 2). Expression is diminished in cortical zone (cz) where hemocytes, labeled here by anti-Peroxidase (Pxn; red) differentiate. F–G: Co-expression of Trol (green) with Laminin A (F,F') and Collagen IV (G,G'), recognized by their respective antibodies (red) in the extracellular matrix of late larval lymph gland. H–J: Electron micrographs of third instar larval lymph gland. Basement membranes surround individual hematopoietic progenitors (ph; small arrow in J) and form irregular septa around groups of cells (large arrow in J. H: Used with permission from Grigorian et al., 2011). Other abbreviations: cb cardioblasts; dv dorsal vessel; lgpl primary lobe of lymph gland; lgsl secondary lobe of lymph gland. Bars: 20 μ m (A–D; E–F); 5 μ m (G).

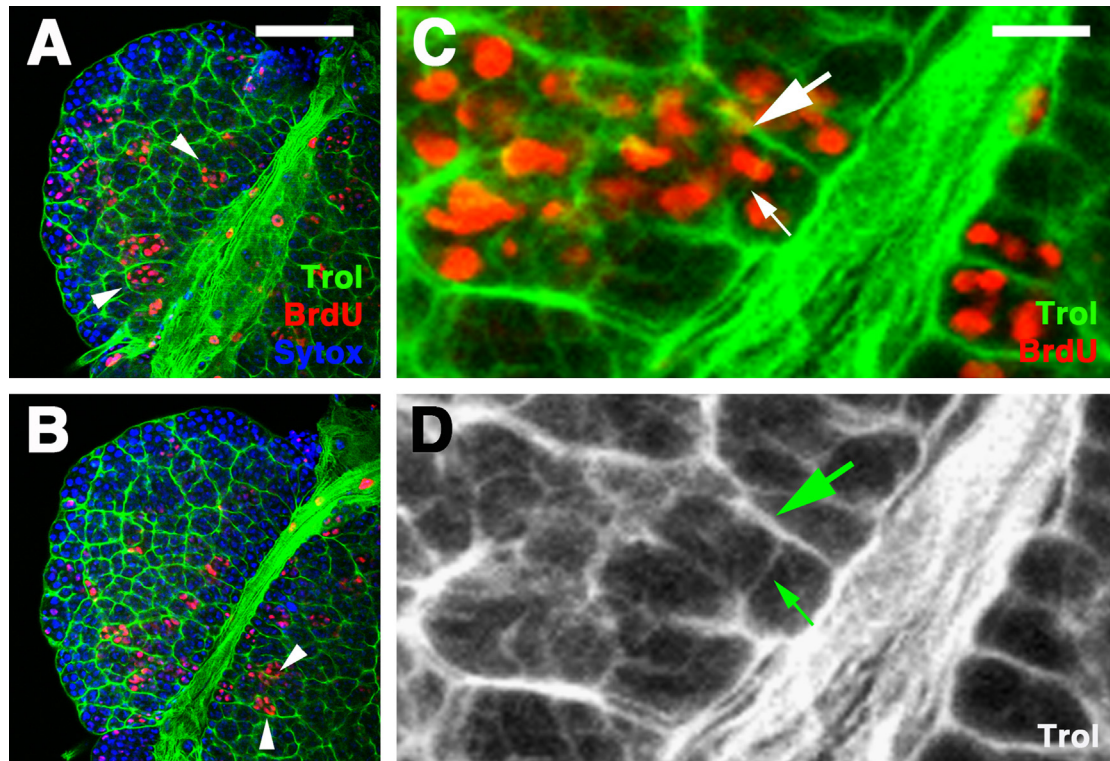


Fig. 2. Pattern of basement membranes in lymph gland is related to hematopoietic progenitor proliferation. A,B: Two confocal section of a ZCL1973X, 72 h larval lymph gland showing expression of *trol* (green), anti-BrdU (red), and Sytox (blue). Larva had been subjected to 1 h pulse of BrdU at 48 h. C,D: High magnification view of BrdU-positive cells (red in C) in relationship to *trol*-positive basement membranes (green in C; white in D). BrdU-positive hematopoietic progenitors form clusters of cells whose boundaries, in most cases, coincide with the septa formed by basement membranes (arrowheads in A,B; large arrows in C,D). Individual cells are surrounded by thin basement membranes (small arrows in C,D). Bar: 40 μm (A,B); 10 μm (C,D).

resulted in a severe reduction of anti-Trol antibody signal in the lymph gland; by contrast, *trol*-RNAi driven by *Cg*-Gal4 and *elav*-Gal4 did not have a significant effect on Trol expression in the lymph gland.

The most conspicuous phenotype of *trol* LOF alleles is a reduced lymph gland size and altered packing of prohemocytes. Light microscopically, the lymph gland of *trol* mutants showed large gaps (lacunae) devoid of cells (arrows in Fig. 4B',D'). A similar phenotype could be observed when expressing *trol*-RNAi ubiquitously by *Tub*-Gal4 (not shown). Laminin and Collagen IV, normally concentrated in thin basement membranes surrounding (groups of) hemocytes (Fig. 4A, C), occupied these lacunae in between strands of cells (Fig. 4B, D). Electron microscopically, these lacunae were solidly filled with a fine-meshed, reticular material (Fig. 4E). At the same time, thin basement membranes around individual cells, or the lymph gland as a whole, were absent. In electron density, the reticular ECM of *trol* mutant lymph glands resembled the basement membranes of wild type (compare panels in Fig. 4F–I). The main difference is best characterized as a change in pattern: in wild type, the macromolecular components of the ECM assemble as a two-dimensional sheet (Fig. 4F–G). In the absence of *trol*, these components are woven into a three-dimensional network of lamellae and fibers (Fig. 4H–I).

Loss of trol results in a reduction of proliferation and premature differentiation of hemocytes

The lymph glands of *trol* mutant larvae are smaller than their wild-type counterparts. Past studies have shown that cell cycle progression in the brain of *trol* mutants is arrested (Caldwell and Datta, 1998). We therefore carried out BrdU incorporation studies in order to assess whether cell proliferation was reduced in *trol*⁸ mutant lymph glands. We found that 48 h and 72 h *trol*⁸ lymph

glands had lower levels of BrdU incorporation in comparison to their WT counterparts (Fig. 5A–D). To verify that the lower numbers of BrdU positive cells seen in *trol*⁸ mutant lymph glands was not due to overall loss in size of the lymph gland, we quantified the percentage of BrdU positive cells in comparison to the overall volume of lymph gland size in mutant and WT 48 h to 72 h lymph glands. Average percentage of BrdU positive cells is 19.18% (SD 5.3%) in *trol*⁸ vs. 44.2% (SD 4.6%) in WT (p value: 0.000186277; t-test: 1 tail, type 2). Since changes in size could also be due to cell death, we carried out an anti-cleaved Caspase-3 staining in mutant lymph glands, however no noticeable cell death was seen (Supplemental Fig. 1).

Significantly, the reduction in proliferation is accompanied by a premature differentiation of hemocytes. Late differentiation markers of plasmacytes, like P1 which is expressed only in the late third instar (96–120 h) of wild type larvae, appeared as early as 72 h post hatching in *trol* mutants (Fig. 5D). Furthermore, in mutant lymph glands, the zonation into a central medullary zone containing undifferentiated hematopoietic progenitors, surrounded by a cortical zone of differentiating hemocytes was abolished. Differentiation markers like P1 (late plasmacytes), Pxn (early plasmacytes), or ProPo (crystal cells) were evenly distributed throughout the lymph gland (Fig. 5E–I, L). Ubiquitous activation of *trol*-RNAi (*tub*-Gal4) had a similarly strong effect in regard to precocious differentiation of hemocytes (Fig. 5M). A milder phenotype could be elicited by knockdown of *trol* via an RNAi construct driven by *dome*-Gal4 (medullary zone; Fig. 5N, O) or *cg*-Gal4 (cortical zone plus fat body; Fig. 5P). Knockdown of *trol* in the cortical zone alone or in the brain had no significant effect on hemocyte differentiation (Fig. 5Q).

The loss of function and knock-down phenotype suggests that the block of differentiation that is normally exerted onto the medullary zone by signals from the PSC and the cortical zone is lifted. To assay for the presence of the PSC we used an antibody

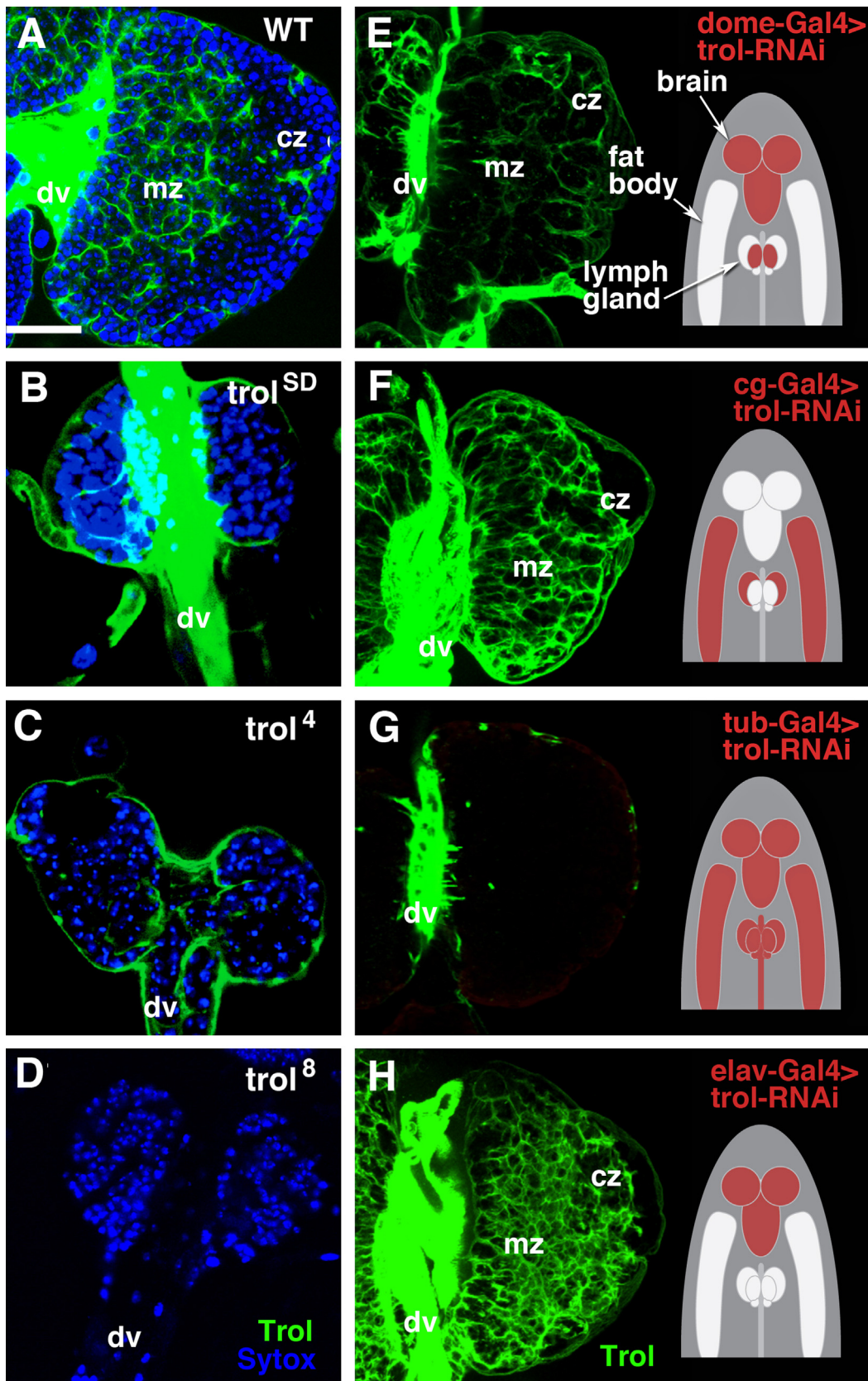


Fig. 3. A–D: Expression of Trol antibody (green) in 96 h wild-type lymph gland (A) and three mutant *trol* alleles: *trol^{SD}* (B), *trol⁴* (C) and *trol⁸* (D). Of the three mutants only *trol⁸* shows a complete lack of anti-Trol antibody staining. E–H: Expression of *trol-RNAi* by four different Gal4 driver lines. Pattern of expression of the drivers (E: *domeless* (*dome*)-Gal4; F: *Collagen* (*Cg*)-Gal4; G: *Tubulin* (*tub*)-Gal4; H: *elav*-Gal4) is shown schematically at right of panels; Trol signal in lymph gland, detected by antibody, is showing at the left. Note that, within the lymph gland, *Cg*-Gal4 is almost exclusively expressed in the cortical zone, even though collagen protein is detected by antibody staining in both cortical and medullary zones; see Fig. 1G,G'. Abbreviations: cz cortical zone; dv dorsal vessel; mz medullary zone; Bar: 20 μ m (A–H).

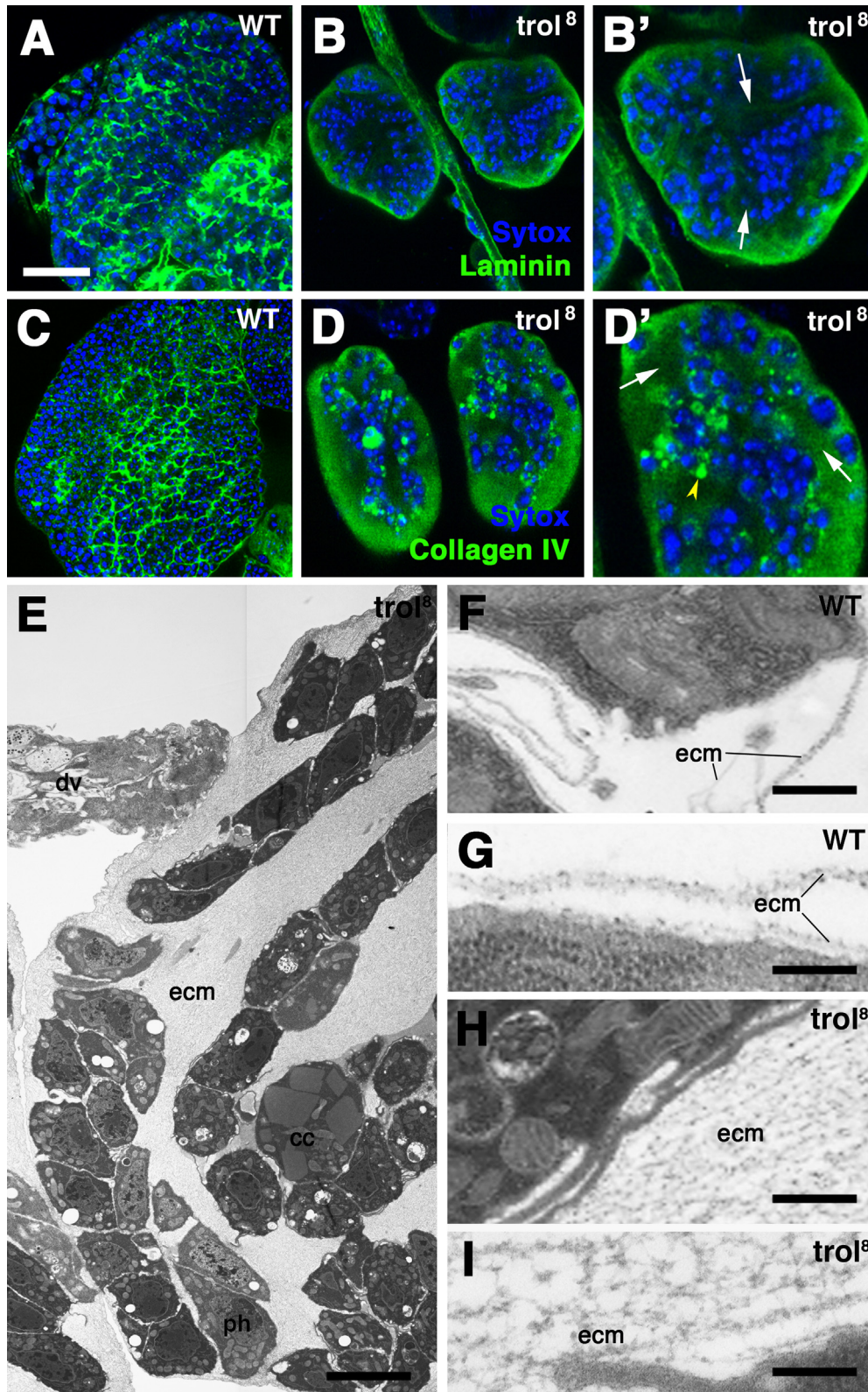


Fig. 4. Loss of *trol* causes structural changes in extracellular matrix of lymph gland. A–D: confocal sections of 96 h wild-type lymph gland (A,C) and *trol*⁸ mutant (B,D) labeled with anti-Laminin (A,B) and anti-Collagen IV antibodies (C,D). B' and D' show higher magnifications of mutant lymph glands presented in B and D, respectively. Laminin and Collagen IV are distributed in thin layers (basement membranes) in wild type; in *trol*⁸ mutants, these proteins solidly fill wide extracellular spaces formed within the lymph gland (arrows in B' and D'). In addition, brightly labeled "puncta" (yellow arrowhead in D') appeared consistently in lymph glands labeled with anti-Collagen IV, but not anti-Laminin. E: Electron micrograph of *trol*⁸ mutant lymph gland. Wide clefts in between clusters of hematopoietic progenitors (ph) are filled with extracellular matrix (ecm). F, G: High magnification electron micrographs of extracellular matrix of wild-type lymph gland, forming thin, two-dimensional basement membranes around cells. H, I: In *trol*⁸ mutant, extracellular matrix is structured as a three-dimensional meshwork of fibers and membranes. Other abbreviations: cc crystal cell; dv dorsal vessel; ph hematopoietic progenitor. Bars: 20 μ m (A–D'); 5 μ m (E); 200 nm (F,H); 100 nm (G,I).

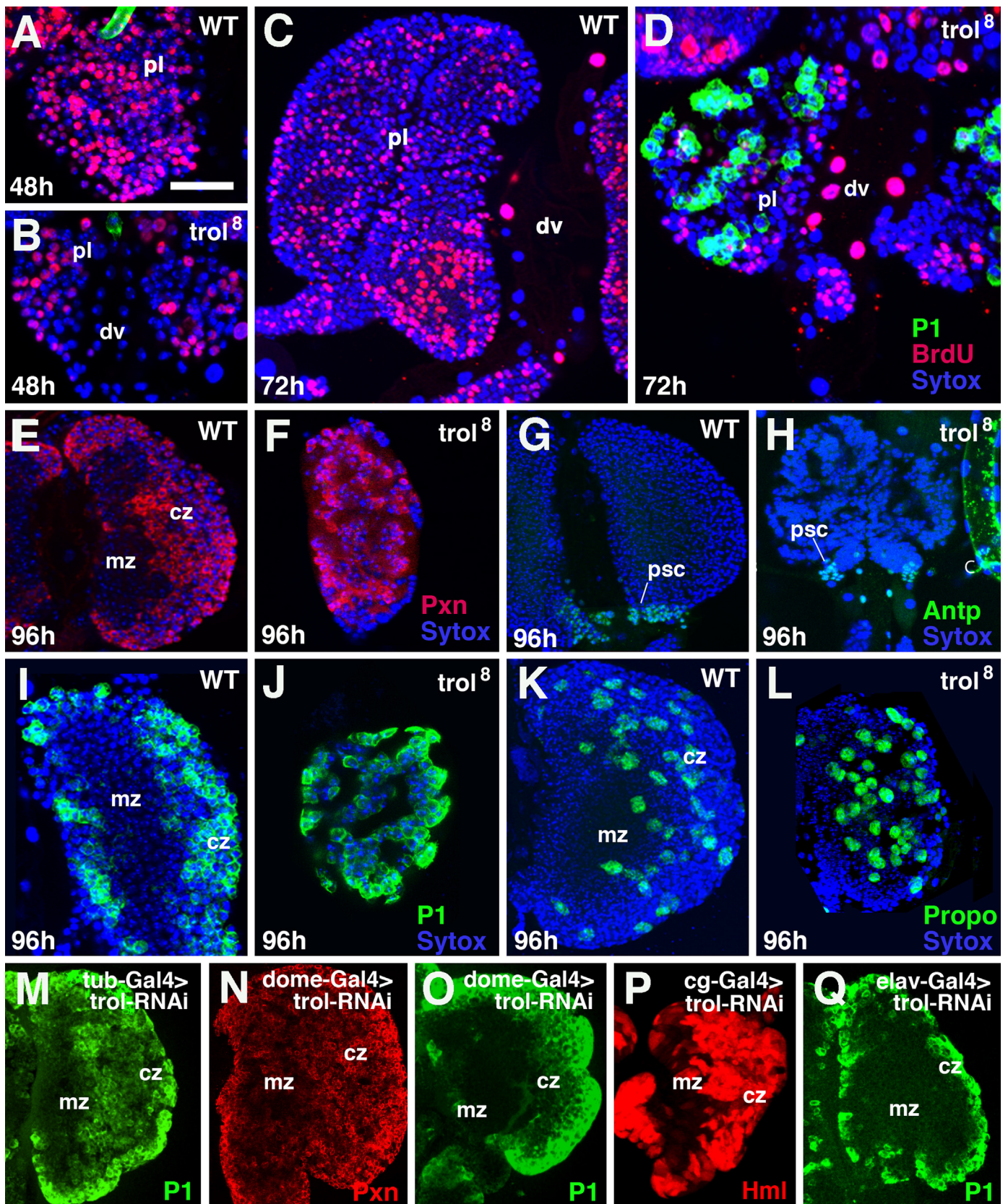


Fig. 5. Loss of *trol* leads to reduced proliferation and precocious differentiation of hematopoietic progenitors. A–D: 1 h pulses of BrdU were applied to wild-type larvae (A,C) and *trol*⁸ mutant larvae (B,D). Larvae were sacrificed at time points indicated (48 h after hatching: A,B; 72 h: C,D). Lymph glands were labeled with anti-BrdU (red) and anti-P1 (green). BrdU-positive cells are strongly reduced in *trol* mutants at both time points. The plasmacytocyte marker P1 is not yet expressed in 72 h wild-type lymph glands (C), but labels clusters of cells in the *trol*⁸ mutant lymph gland (D). E–L: Expression of hemocyte differentiation markers in lymph glands of 96 h wild-type and *trol*⁸ mutant larvae. In 96 h wild-type lymph glands, early hemocyte marker peroxidase (Pxn, red in E), late plasmacytocyte marker P1 (green in I) and crystal cell marker Propo (green in K) are restricted to the cortical zone (cz). These markers are evenly expressed throughout the lymph glands of 96 h *trol*⁸ mutants (F,J,L). Antp, a marker for the PSC (green in G,H) is expressed normally in the *trol*⁸ mutant. M–Q: Driving a *trol*-RNAi construct with different driver lines [*Tubulin* (*Tub*)-Gal4 (M), *dome*-Gal4 (N,O), *Collagen* (*Cg*)-Gal4 (P), *elav*-Gal4 (Q)] results in precocious expression of differentiation markers (M,N,P; P1: green; Peroxidase (Pxn) or Hemolectin (Hml): red). Hml expression seen via a reporter line (*Hml*Δ-DsRed) crossed into the stated background. Bar: 20 μm (A–Q). Other Abbreviations: cz cortical zone; mz medullary zone; dv dorsal vessel; pl primary lobe; psc posterior signaling center.

against Antp (Mandal et al., 2007). The expression of this marker remained unchanged (Fig. 5G,H) in regard to level and cell number, indicating that the premature differentiation of the medullary zone is not the result of a lack of the PSC.

Loss of *trol* interferes with the Hh signaling pathway

Hh signaling plays an important role in determining hemocyte differentiation within the lymph gland. Hh signals originating from the PSC maintain cells of the medullary zone in an undifferentiated state (Mandal et al., 2007). Loss of this signal results in mass differentiation of the cells of the lymph gland. *Trol* is known to play a role in modulating molecular signals, including Hh. Since we had observed a phenotype where the cells of the *trol*⁸ mutant lymph glands were differentiated regardless of their location in a medullary or cortical zone, we decided to look into whether *trol* may be effecting Hh signaling.

The expression level of the receptor Patched (*Ptc*), which is also utilized as a read-out for Hh-signaling activity, was reduced in the medullary zone (Fig. 6A,C). The transcription factor *Cubitus interruptus* (*Ci*), which is activated by Hh signaling from the PSC, and is normally expressed in the medullary zone (Mandal et al., 2007), was also strongly reduced or absent in the *trol*⁸ mutant (Fig. 6B,D). Both findings indicate that in the absence of *trol*, Hh signaling to the medullary zone is diminished. Genetic interaction between *hh* and *trol* further support this notion. We used the heat-shock inducible *hs-hh* construct (Ingham, 1993; Park et al., 2003) in the background of homozygous *trol* larvae to test the prediction that overexpression of *hh* will rescue the *trol* hemocyte differentiation phenotype in the lymph gland. Expression of the *hs-hh* construct alone in wild-type larvae caused a delay in hemocyte differentiation compared to WT (Fig. 6E–F). This was expected based on past studies that have shown that Hh acts to maintain lymph gland hematopoietic progenitors in an undifferentiated state (Mandal et al., 2007). Expressing *hs-hh* in the background of *trol* LOF animals rescued the global, premature differentiation of primary lobes characteristic for the *trol* mutant (compare Fig. 6G and H,I). Lymph glands were still reduced in size and showed wide, cell-free gaps, but contained no differentiated cells in their central domain (“medullary zone”). P1 was almost entirely absent; Pxn was expressed in clusters of hemocytes restricted to the periphery (“cortical zone”) of the lymph gland (data not shown). These results suggest a model where the Hh signal, in order to act on cells of the medullary zone, requires the presence of the ECM protein *Trol*. Loss of *Trol* renders endogenous Hh ineffective; however, by applying Hh in excess by utilizing *hs-Hh*, its role as an inhibitor of differentiation can be partially restored.

To further test an interaction between *Trol* and Hh, *trol*⁸ and *hh*^{AC} lines were crossed and progeny heterozygous for both alleles were examined to view a possible amplification of phenotype. Using P1 as a differentiation marker, primary lymph glands of heterozygous *trol*⁸, *hh*^{AC} and *trol*⁸/*hh*^{AC} were examined. All heterozygote mutants had mild precocious differentiation within the primary lobes. However, a marked amplification of the phenotype was observed in the secondary lobes of the double heterozygotes over the simple heterozygotes. In WT animals, secondary lobe cells of the pupa disperse before expression of P1

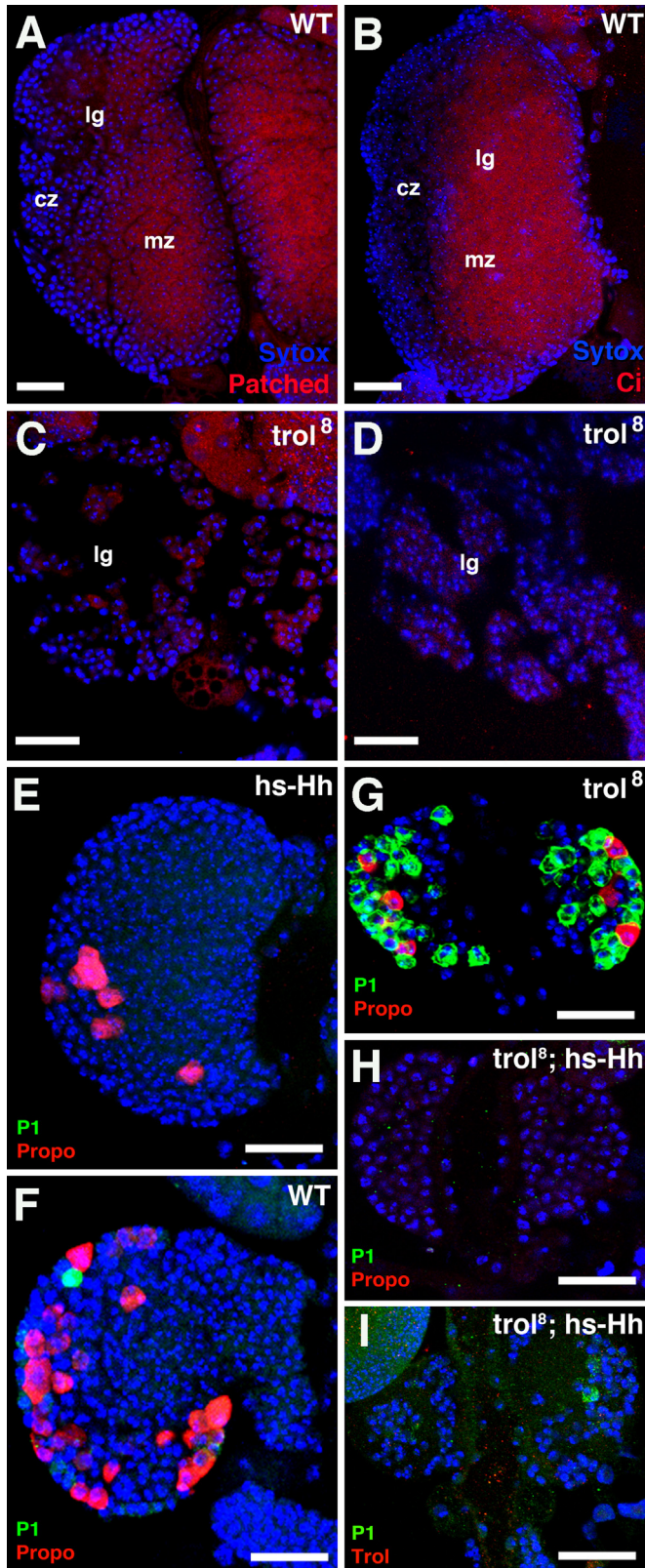


Fig. 6. Genetic interaction between *trol* and Hh signaling pathway members. A–D: Genes activated by Hh signaling, *patched* (*ptc*; A) and *cubitus interruptus* (*ci*; B) are expressed in medullary zone (mz) of 96 h wild-type lymph gland (*Ptc* and *Ci* proteins recognized by antibodies; red). Expression levels of these genes are strongly reduced in *trol*⁸ mutant (C,D). E–I: Precocious differentiation of hemocytes in *trol* can be rescued by ectopic *hh* expression. All panels show lymph glands of late third instar larvae which have been heat shocked (see Material and Methods). E: In wild-type lymph glands, hemocyte differentiation is initiated in the cortical zone, as shown by expression of the crystal cell marker *Propo* (red) and the late plasmatocyte marker *P1* (green). F: Larvae expressing *hs-hh* in a wild-type background show reduced hemocyte differentiation in comparison to the WT. G: In a *trol*⁸ mutant that has been heat shocked, both *Propo* and *P1* are expressed throughout the lymph gland. H,I: Expression of *hs-hh* in the background of *trol*⁸ (note absence of *Trol* protein shown in I) prevents hemocyte differentiation. Bar: 20 μm (A–E). Other abbreviations: cz cortical zone; lg lymph gland. Bars: 20 μm.

(Grigorian et al., 2011). In contrast, while the secondary lobes of *trol*⁸ and *hh*^{AC} heterozygotes showed few P1 positive cells, the secondary lobes of *trol*⁸/*hh*^{AC} double mutants showed a marked increase in P1 positive cells (Supplemental Fig. 2).

Discussion

Perlecan/Trol is a secreted heparin sulfate proteoglycan (HSPG) that forms a widespread component of the ECM. Members of the HSPG family are comprised of a protein core to which heparin sulfate glycosaminoglycan chains are attached (Bernfield et al., 1999; Esko and Selleck, 2002; reviewed in Yan and Lin, 2009). A number of genes encoding glycosyltransferases and modification enzymes that include *tout-velu* (*ttv*), *sister of ttv* (*sotv*), *brother of ttv* (*botv*) and *sulfateless* (*sfl*) are conserved from vertebrates to *Drosophila*, and are important for the polymerization and modification of heparin sulfate chains during their biosynthesis (Esko and Selleck, 2002; Nybakken and Perrimon, 2002; Lin and Perrimon, 2002; reviewed in Yan and Lin, 2009). Loss of these enzymes has been shown to lead to defective morphogen signaling and gradient formation, showing the importance of heparin sulfate chains for the role of HSPGs in the ECM.

Our study of *Drosophila* Perlecan/Trol shows that this protein plays an important role in *Drosophila* hematopoiesis. Trol is expressed in the hematopoietic organ, the lymph gland, from embryonic stages onward. As shown in our previous work (Grigorian et al., 2011) and in this paper, the ECM of the lymph gland forms thin lamellae (“basement membranes”) surrounding individual hemocytes, as well as the organ as a whole. A similar organization of the ECM has also been noted in the hematopoietic organs of other insects (Hoffmann et al., 1979; Nardi et al., 2003; Grigorian et al., 2011). For example, in *Locusta migratoria*, Hoffmann et al. (1979) describe “fine lamellae of basement membrane” around the hemocytes. The basal laminae within the *Manduca* hematopoietic organ also seem to form chambers around hemocytes; Nardi et al. (2003) describe groups of contiguous cells within the hematopoietic organ that appear to be tightly held together by basal laminae, thereby somewhat separating them from more loosely associated single cells that are also found in the organ. As hemocytes move from the hematopoietic organ into circulation, they locally break through this ECM (Grigorian et al., 2011). Similarly, in *Manduca sexta*, the basal lamina surrounding the hematopoietic organ shows gaps in the domain where hemocytes are released (Nardi et al., 2003).

Drosophila trol mutants exhibit a complex lymph gland phenotype. We speculate that this phenotype is caused by (1) the role of Trol as a structural component of the ECM, and (2) its role as a binding partner to extracellular signals, such as Hh. In regard to the first role, we observed an anomalous ultrastructure of the lymph gland ECM, which forms a sponge-like, three dimensional mesh, as opposed to the two-dimensional lamellae seen in the wild type. Correspondingly, the distribution of Laminin and Collagen IV is also affected in *trol* mutants. Instead of forming the lamellae characteristically seen in wild type, Collagen IV and Laminin are distributed diffusely throughout the wide lacunar spaces that riddle the *trol*-mutant lymph gland. This finding suggests that Trol exerts an organizing effects on other constituents of the ECM, such as Laminin and Collagen. Converse studies, looking at Trol protein in the background of deficiencies of other ECM components, have revealed that a mutation in *laminin* β 1 chain causes a loss of both Collagen IV and Trol accumulation around the cardioblasts of *D. melanogaster* embryos (Urbano et al., 2009). Collagen IV mutants in *D. melanogaster* also lose Trol in the basement membranes of embryos and larval wing

discs (Pastor-Pareja and Xu, 2011). A recent study (Pastor-Pareja and Xu, 2011) investigated the function of *trol* during wing disc development using *trol*-RNAi. This treatment did not cause the loss of Collagen IV or Laminin. Structurally, *trol*-RNAi reduced the thickness of the wing disc basement membrane. The mesh-like ECM phenotype that we witnessed in the *trol*⁸ mutant larval lymph gland was not observed in the wing disc. In the lymph gland, knocking down *trol* via *dome*-RNAi also causes only slightly premature expression of the hemocyte differentiation markers Pxn and P1, but does not have an apparent effect on general ECM structure (this study; Dragojlovick-Munther and Martinez-Agosto, this issue). On the other hand, driving the *trol*-RNAi construct ubiquitously (*Tub*-Gal4) results in a much stronger phenotype, pointing to the importance of tissues outside the lymph gland in shaping the ECM surrounding hemocytes and their progenitors. Previous work has shown that the basement membranes surrounding a number of larval structures, notably the imaginal discs, are assembled from ECM produced in the fat body, rather than the epithelial cells of the discs themselves (Pastor-Pareja and Xu, 2011). It is likely that the basement membranes surrounding hemocytes in the lymph gland may also be enriched by proteins diffusing from the fat body, via the hemolymph, into the glands. The lack of any lymph gland phenotype when knocking down Trol in the brain (*elav*-Gal4) demonstrates that general neurological abnormalities, which might have an indirect effect on growth and metabolism, did not account for the strong lymph gland phenotype seen in *trol* LOF alleles and RNAi knockdowns.

In vertebrates, Perlecan binds to Laminin and Collagen (Whitelock et al., 2008) and is known to play a role in Laminin organization in mouse embryonic stem cells (Henry et al., 2001). Perlecan is also involved in cartilage matrix assembly and has been seen to promote collagen fibril formation (Kvist et al., 2006). On the organismic level, mutations in vertebrate Perlecan have a profound effect on blood and cardiovascular development. Morpholino-mediated knockdown of Perlecan in zebrafish showed abnormal development of the angiogenic intersegmental and dorsal longitudinal anastomotic vessels (Zoeller et al., 2008). Knock-out of Perlecan in mice causes a pleiotropic phenotype (Arikawa-Hirasawa et al., 1999; Costell et al., 1999; reviewed in Whitelock et al., 2008), reflecting the fact that Perlecan is expressed in many different tissues throughout development. In the cardiovascular system, Perlecan KO mice show massive blood leakage into the pericardial cavity (Sasse et al., 2008). Further vascular defects include abnormal coronary vessels, as well as transposition of the great arteries (Hassell et al., 2002). Perlecan is also a component of the ECM in bone marrow (Klein et al., 1995), where it is expressed strongly between maturing hematopoietic cells, as well as in the basement membrane of endothelial cells. Perlecan is thought to interact closely with proliferating and differentiating hematopoietic cells. Interestingly, in vitro cell attachment assays indicate that Perlecan appears to reduce the adhesion of hematopoietic cells (Klein et al., 1995). This role as a repellent for hematopoietic cells may be important for the regulation of cell migration and egress into circulation.

Aside from the structural defects, loss of function in Perlecan or other glycoproteins causes defects that are attributable to disruptions in signaling mechanisms. These observations emphasize the (permissive) role of ECM components in shaping the pattern of release and the concentration gradients of secreted signals. We report here that the lymph glands are significantly smaller in size than their WT counterparts due to reduced cell divisions. This phenotype is reminiscent of the reduced proliferation in larval brain neuroblasts that gave the *trol* mutation (“terribly reduced optic lobes”) its name (Datta and Kankel, 1992). In the larval brain, *trol* promotes cell cycle progression of neuroblasts via the FGF and

Hh signaling pathways (Park et al., 2003; Caldwell and Datta, 1998; Lindner et al., 2007). Datta and colleagues also noted a significant drop in the number of circulating larval hemocytes in the *trol* mutant (Lindner et al., 2007), and speculated that *trol* promotes Ras-MAPK via the Pvr (PDGF/VEGF) signaling pathway, which has been shown to play a role in hemocyte maintenance in the embryo (Bruckner et al., 2004). It is conceivable that the reduced proliferation in the *trol* mutant larval lymph gland described in this paper is also mediated via an effect of Trol on PVR signaling. Published findings demonstrate that PVR signaling plays a role in relaying a signal between the PSC and the cortical zone that is important in maintaining cells of the medullary zone in an undifferentiated state (Mondal et al., 2011). No data exist to date to settle an earlier role of PVR in prohemocyte proliferation during the early larval stage. However, FGFR signaling plays a role in this process, and interacts with Trol (Dragojlovic-Munther and Martinez-Agosto, this issue).

A further characteristic phenotype of the *trol* mutation is precocious differentiation of the hematopoietic progenitors of the lymph gland. Whereas P1 expression is seen by 72 h in the *trol*⁸ mutant, WT lymph glands do not show expression of this marker until 96 h. In addition to its early onset, differentiation is not limited to the cortical zone of the lymph gland, as is seen in WT, but occurs in the medullary zone as well. In the lymph gland of *D. melanogaster* larvae, Hh is known to play a role in maintaining the cells of the medullary zone in an undifferentiated state (Mandal et al., 2007). Our findings indicate that the precocious and disordered differentiation observed in *trol* lymph glands is indeed due to a reduced activity in Hh signaling, prompting the hypothesis that Hh, secreted from the PSC, requires Trol and/or an intact ECM to act upon the medullary zone. Previous studies have shown that Perlecan can bind Hh, Wnt and FGF, thereby controlling the concentration of these molecules in the pericellular space and the amount of signal that is received by cells (reviewed in Iozzo, 2005; Whitelock et al., 2008). In other words, Perlecan and other glycoproteins of the ECM shape the gradient of Hh and other morphogens. In the wing disc, mutant clones of the glycosyltransferase *ttv* exhibit impaired Hh signaling. Hh appears to be unable to diffuse past these mutant cells (Bellaiche et al., 1998). Clones carrying a double mutation for the *Drosophila* HSPG glypicans *division abnormally delayed* (*dally*) and *dally-like-protein* (*dlp*) show a similar, albeit weaker phenotype to that seen for *ttv* (Han et al., 2004; reviewed in Yan and Lin, 2009). Furthermore, co-immunoprecipitation studies in third instar larvae of *D. melanogaster* demonstrate a direct interaction between Trol and Hh (Park et al., 2003). Lipid-modification of Hh is needed for its interaction with HSPGs (Bellaiche et al., 1998; Callejo et al., 2006; Gallet et al., 2006; reviewed in Yan and Lin, 2009). Another way of picturing the involvement of Trol in Hh signaling is to view the ECM as a physical conduit for the normal spreading of Hh. Even under normal circumstances, ECM acts as a migration and diffusion barrier (Korpos et al., 2010; Sabeh et al., 2004). The altered state of the ECM in *trol* mutant lymph glands may further inhibit proper Hh dispersion. We propose that the *Drosophila* larval lymph gland, with its simple structure and highly characteristic lamellar ECM architecture, represents a favorable system to approach genetically the role of ECM in cell–cell signaling pathways.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.03.007>.

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