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ORIGINAL ARTICLE

discs large regulates somatic cyst cell survival and expansion in *Drosophila* testis

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Gonad development requires a coordinated soma-germline interaction that ensures renewal and differentiation of germline and somatic stem cells to ultimately produce mature gametes. The *Drosophila* tumour suppressor gene *discs large* (*dlg*) encodes a septate junction protein functioning during epithelial polarization, asymmetric neuroblast division, and formation of neuromuscular junctions. Here, we report the role of *dlg* in testis development and its critical function in somatic cyst cells (SCCs). In these cells *dlg* is primarily required for their survival and expansion, and contributes to spermatocyte cyst differentiation. Cell death primarily occurred in SCCs at the end of spermatogonial amplification at a time when Dlg becomes restricted in wild-type (wt) testes to the distal somatic cells capping the growing spermatocyte cysts. RNAi depletion of *dlg* transcripts in early SCCs fully prevented testis development, whereas depletion in late SCCs resulted in a breakdown of spermatocyte cyst structure and germ cell individualization. Specific *dlg* expression in SCCs resulted in developmental rescue of *dlg* mutant testes, whereas its expression in germ cells exerted no such effect. *dlg* overexpression in wt testes led to spermatocyte cyst expansion at the expense of spermatogonial cysts. Our data demonstrate that *dlg* is essentially required in SCCs for their survival, expansion, and differentiation, and for the encapsulation of the germline cells.

***Keywords*:** *discs large*, *Drosophila*, testis formation, somatic cyst cells
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Introduction

In *Drosophila melanogaster* the development of embryonic gonad results from the coalescence of two specialized cell types, migrating germ cells, and somatic mesodermal cells [1]. Following gonad coalescence during which the germ cells are surrounded by somatic mesodermal cells extending cellular processes between them, the gonad becomes compacted and spherical [2]. The development of male and female gonads already differs at the time of gonad coalescence, as male-specific somatic mesodermal cells join the posterior of the male gonad, while the equivalent cells die by apoptosis in females [2]. A hub is formed at the anterior of the late embryonic male gonad and consists of a cluster of non-dividing apical mesodermal cells, which organize the anterior-most germ cells in a rosette arrangement similar to that of germline stem cells (GSCs) in the adult [2]. The first signs of testis organogenesis are detected in first instar larvae (L1) when the germ cells start to expand, whereas those of the ovaries remain quiescent [2, 3].

In the niche, the GSCs enclosed in a pair of somatic stem cells (SSCs), are physically attached to the hub cells (Figure 1A) via adherens junctions [4]. Each GSC divides asymmetrically with the mitotic spindle orientated perpendicular to the hub [5, 6]. One of the daughter cells remains in contact with the hub, inherits the mother centriole, and retains GSC identity, whereas the other daughter cell, called gonialblast, inherits the daughter centriole. This cell is displaced from the hub and initiates differentiation [7-9]. The gonialblast, wrapped by two somatic cyst cells (SCCs) [10], divides mitotically four times to give rise to a cyst of 16 interconnected spermatogonial cells. The SCCs grow without division, become flat, elongate, and form a thin layer around the spermatogonial cyst (SC). The germ cells become transcriptionally highly active and form spermatocytes,

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Abbreviations: *dlg* (*discs large*); GSCs (germline stem cells); L (larval stage); *lgl* (*lethal (2) giant larvae*); SCs (spermatogonial cysts); SCCs (somatic cyst cells); *scrib* (*scribble*); SSCs (somatic stem cells); wt (wild type)
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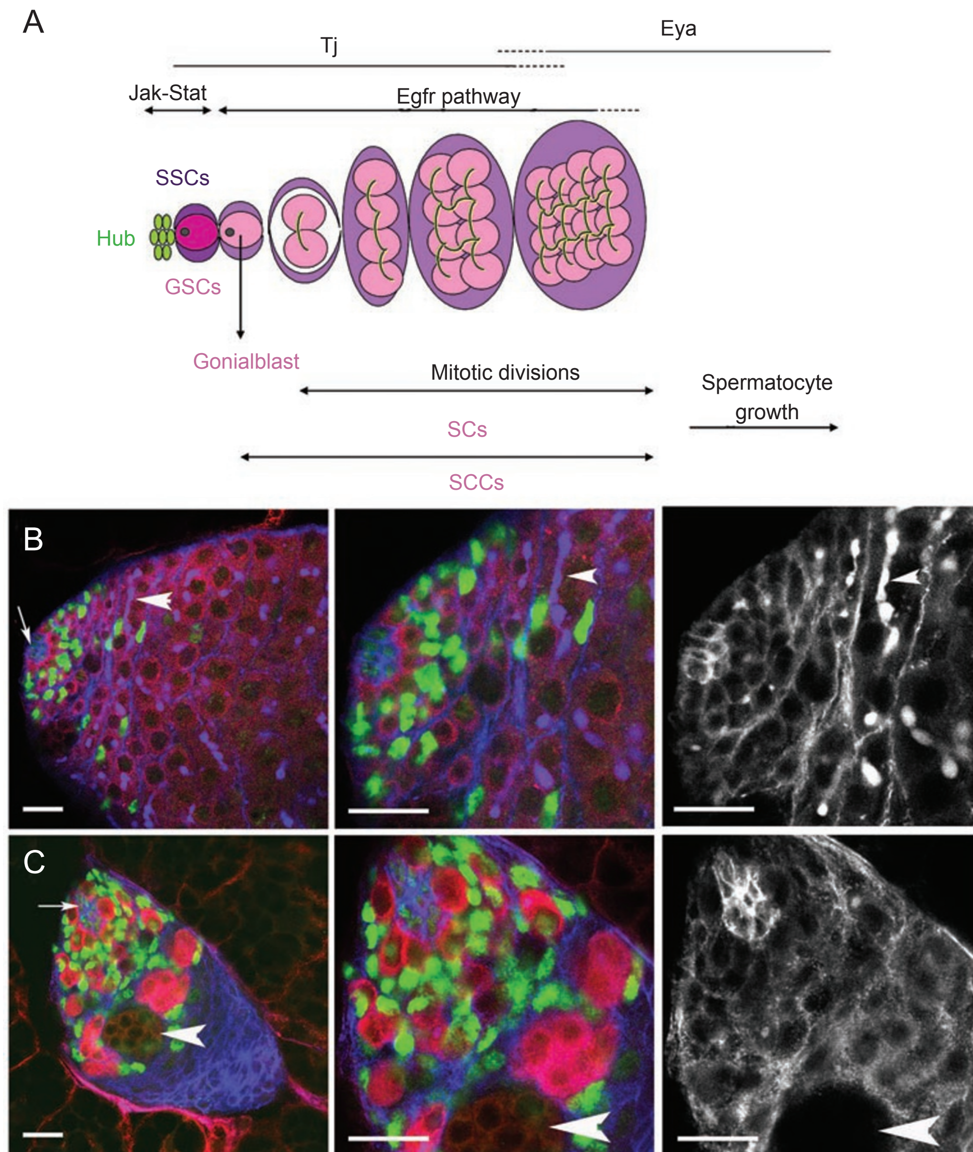

Figure 1 Reduced number of GSCs and SCs in *dlg* testis. **(A)** Diagram depicting early spermatogenesis. Eya, Eyes absent; GSCs, germline stem cells; SC, spermatogonial cyst; SCCs, somatic cyst cells; SSCs, somatic stem cells; Tj, traffic jam. The black dots in GSCs and goniablasts and the black-yellow arches in spermatogonial cells represent the spectrosome and the fusome, respectively. Testes from 5-day-old **(B)** wt and **(C)** *dlg* L3 larvae stained for Vasa (red), Tj (green), and Arm+α-Spectrin (blue). Mid panel pictures are enlargements of the hub region shown on left, whereas the right panel pictures show co-staining of Arm and α-Spectrin. Testis hub (arrow) is oriented towards the left. Arrowheads in **(B)** point out the fusomes and arrowheads in C indicate a weak Vasa-stained SC. Bar: 15 μm.

which over a period of 80 h will considerably increase in size and ultimately undergo meiosis [11].

The balance between stem cell identity and differentiation results from signals exchanged between the hub, GSCs and SSCs. The hub cells secrete Unpaired (Upd), which activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway in adjacent GSCs. In absence of JAK-STAT signaling the GSCs differentiate and are unable to undertake self-renewal. Conversely, increased expression of JAK-STAT greatly expands the population of GSCs and SSCs [12, 13]. A further signaling pathway restricting GSC proliferation is mediated by the epidermal growth factor receptor (Egfr), whose inactivation in SSCs leads to an expansion of male GSCs [14]. Similarly, Raf, an Egfr downstream component, is required in SSCs to limit GSC expansion [15]. In testes mutated for the *rhomboid* homologue *stet*, the germ cells fail to associate with SSCs [16]. Further-

more, germ cells recruit SSCs via the ligand Spitz, which binds to Egfr, and acts through the nucleotide exchange factor Vav to regulate the activity of Rac1, a downstream component of the Egfr pathway [17]. In addition, loss of the TGF-β signal transducers *punt* and *schnurri* in SCCs leads to overproliferation of gonialblasts and spermatogonial cells but not of GSCs [18].

The observation that three well-established tumor suppressor genes, *discs large* (*dlg*) [19, 20], *scribble (scrib)* [21, 22], and *lethal (2) giant larvae (lgl)* [23], are expressed in the *Drosophila* embryonic gonads and larval testis [24], prompted us to analyze their function in this tissue. Here, we report that *dlg* plays a critical role in testis formation during larval development primarily by regulating somatic cell survival.

Results

Reduced number of GSCs and SCs in dlg larval testes

As *dlg* testes were particularly small (both amorphic *dlg*^{m52} and hypomorphic *dlg*^{X12} mutations led to a similar phenotype), we examined which types of cells were affected by *dlg* inactivation. Similar to wt (wild type) (Figure 1B) the hub of a *dlg* testis (Figure 1C) appeared normal and consisted of tightly packed cells positively stained for Armadillo (Arm), suggesting that adherens junctions remained intact in the hub region. In contrast, the number of GSCs was reduced in *dlg* (4.9 GSCs/testis in 12 testes examined) with respect to wt (8.1 GSCs/testis in eight testes examined). Furthermore, the *dlg* GSCs were less tightly packed around the hub. Compared to *dlg*, *scrib* testes showed no sign of degeneration with the exception that they were reduced in size, whereas *lgl* testes were comparatively normal in size and shape (data not shown). The size of *dlg* GSCs, gonialblasts, and spermatogonial cells were larger than in wt testes. Graphical determination of the length (L) and width (W) of wt ($L = 6.2 \pm 1.0$ μm, $W = 4.9 \pm 0.4$ μm) and *dlg* ($L = 9.5 \pm 3.3$ μm, $W = 6.5 \pm 0.88$ μm) GSCs revealed that the volume of the *dlg* cells should be two to three times larger than that of wt GSCs. Moreover, *dlg* germ cells showed a more intense staining of the germ cell-specific marker Vasa, suggesting an accumulation of Vasa proteins in these cells. In addition, we found a marked reduction in the number of 8- or 16-cell cysts in L3 *dlg* testes. Surviving cysts showed Vasa staining, stronger than in wt. However, few large 16-cell cysts exhibited a much weaker Vasa staining (Figure 1C, arrowhead), suggesting that these cysts were engaged in an apoptotic process. A further characteristic of spermatogonial cells was the gradual disappearance of spectrosomes and fusomes [4, 25], which were absent in L3 *dlg* testes (right in Figure 1B and C), albeit present in *scrib* testes (data not shown). These data indicate that multiple defects occurred during early spermatogenesis, leading to SC disappearance and testis atrophy.

As gonad development requires a coordinated soma-germline interaction ensuing renewal and differentiation of germline and SSCs, we first studied the distribution of known nuclear markers of somatic cell differentiation, including Traffic-jam (Tj) [26] and Eyes absent (Eya) [27]. In wt testes, Tj is produced in early SCCs and in elongated SCCs wrapping 2-, 4-, 8-cell cysts (Figure 1B), whereas Eya is present in SCCs enclosing larger cysts (Figure 2A) [28]. In *dlg* testes of 3-day-old larvae (Figure 1C), we found that the number of positively stained Tj cells was comparable to that in wt (Figure 1B). In contrast, the number of Eya-positive cells was

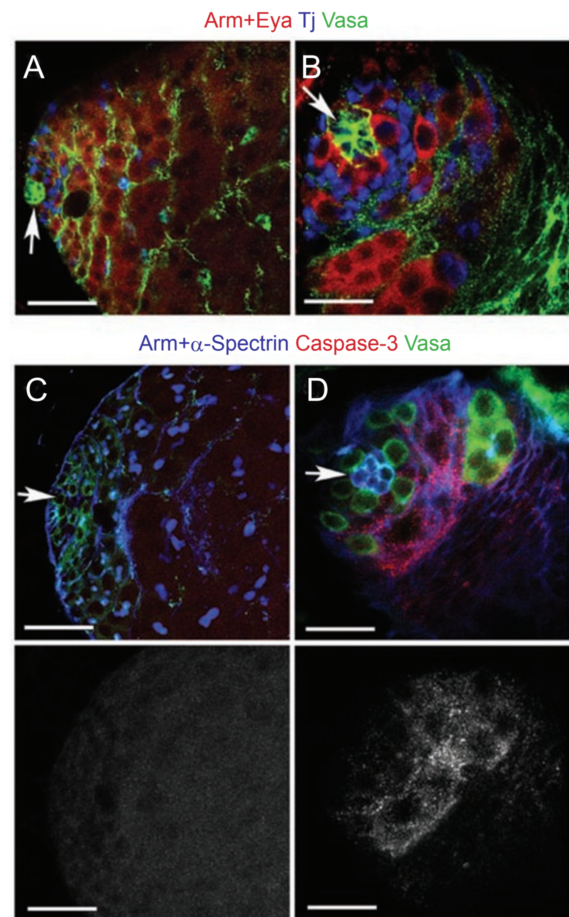

Figure 2 Cell death takes place primarily in late SCCs. **(A)** wt and **(B)** *dlg* testes were stained for Vasa (red), Tj (blue), and Arm+Eya (green). **(C)** wt and **(D)** *dlg* testes stained for activated caspase-3 (red), Vasa (green), and Arm+α-Spectrin (blue). Lower panel pictures show the caspase-3 staining. Testis hub (arrow) is oriented towards the left. Bar: 15 μm.

strongly reduced in *dlg* (Figure 2B). These data suggest a developmental arrest occurring when SCs are wrapped by SCCs. Therefore, we determined which type of cells displayed the first sign of cell death by staining *dlg* testes with rabbit monoclonal anti-active caspase-3 antibody and found that cell death took place predominantly in SCCs and also affected germ cells in the largest 8- and 16-cell cysts (Figure 2D). Similar results were obtained by using the TUNEL assay (data not shown). Expression of a *UAS-p35* transgene encoding a baculovirus homolog of the *Drosophila* inhibitor of apoptosis protein [29, 30] in the germ line and somatic cells to block caspase activation had no gross effect on the size of *dlg* testes (data not shown), suggesting a cell death pathway independent of *Diap*. Although the exact mechanism by which cell death takes place in *dlg* testes remains to be further determined, our data led us to conclude that *dlg* function is primarily required in SCCs for their survival and indicate that a somatic *dlg* function is needed for the expansion and differentiation of germ cells.

The marked degeneration of *dlg* testis incited us to determine more precisely when the first abnormalities can be detected during testis morphogenesis. As shown in Figure 3, *dlg* testes already showed signs of degeneration in L1 larvae. These testes were characterized by a smaller number of germ cells, predominantly aggregated around the hub or in its vicinity. In L2 mutant larvae, the defects became more obvious with few GSCs attached to the hub and a small number of larger SCs.

Dlg distribution in differentiating cysts

To ascertain the role of *dlg* in SCCs, we investigated the pattern of Dlg distribution during normal testis development. In L1 testis (Figure 4A), Dlg was uniformly associated with the cytoskeletal matrix of all somatic and germ cells, and its distribution overlapped with that of F-actin (data not shown). In L2 (Figure 4B) and L3 testes (Figure 4C-F), we observed a uniform Dlg distribution among the hub cells, in between SSCs and GSCs associated with the hub and at the interphase of SCCs and germ cells in the newly formed 2-, 4-, and 8-cell SCs (Figure 4C-E). However, the pattern dramatically changed within the 16-cell SCs in which Dlg decorated the distal SCC. In this cell the Dlg staining was associated with the plasma membrane forming a relatively smooth line with short indentations at contact sites with neighbouring germ cells (Figure 4F). This pattern was limited to a crescent capping a portion of the somatic cell located near the surface of the testis. In more internal locations, Dlg was essentially present at junctions between cyst cells. As shown in Figure 4G-J co-immunostaining with anti-Dlg and anti-Eya revealed that the Eya-positive nuclei (arrows)

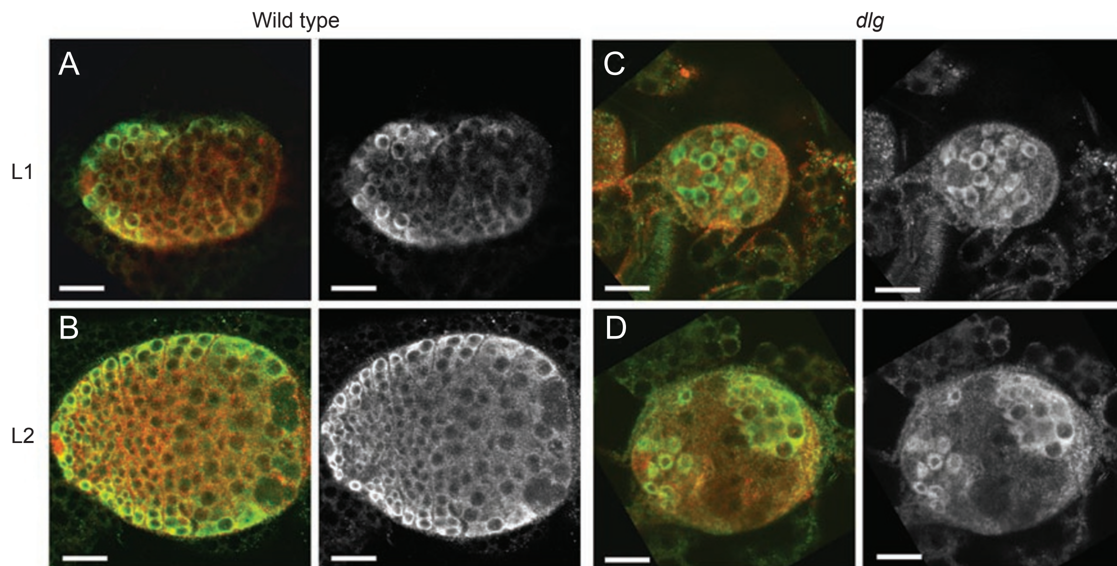

Figure 3 Reduced size of *dlg* testes and reduced number of germ cells. **(A**, **B)** Wt and **(C**, **D)** *dlg* testes from **(A**, **C)** L1 and **(B**, **D)** L2 larvae. Cell contours were visualized by Scribble I (red) and germ cells were detected by using Vasa antibodies (green). Testis hub is oriented towards the left. The size of *dlg* testes was smaller than in wt and depleted in germ cells as detected with Vasa antibodies. In *dlg* testes, germline stem cells (GSCs) were loosely attached to the hub and the overall number of germ cells and SCs were markedly reduced in comparison to wt testes. Pictures on the right of **(A**-**D)** display the Vasa staining. Bar: 15 µm.

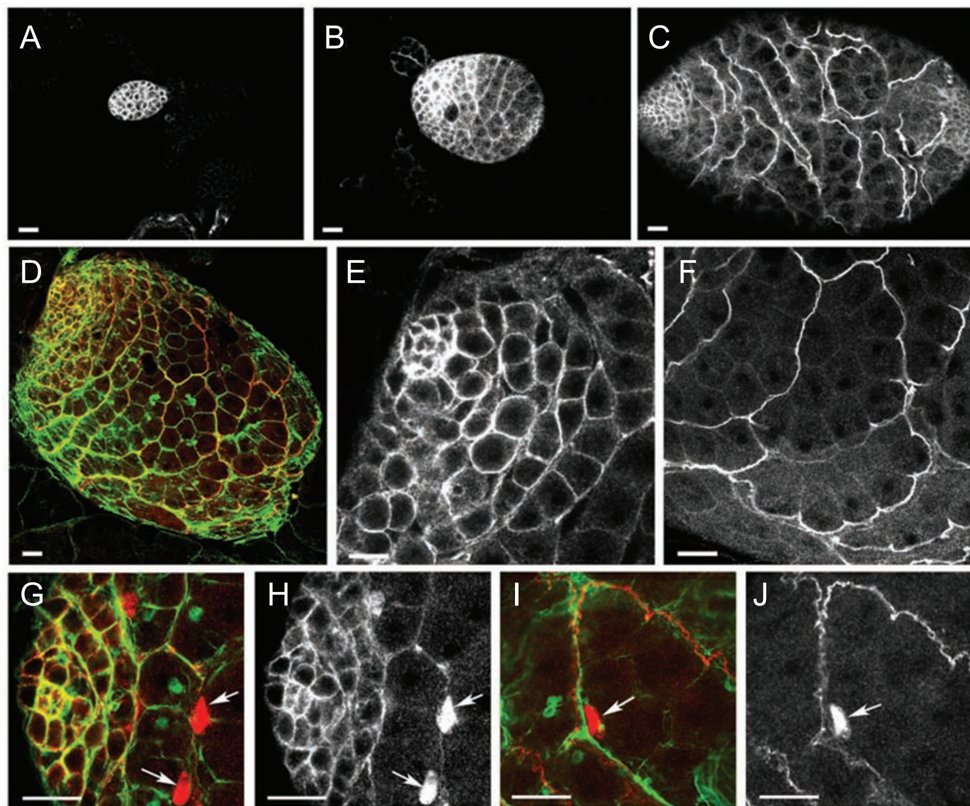

Figure 4 Pattern of Dlg distribution in testis during larval development. Wt testes stained for Dlg in **(A)** L1 (36 h after egg laying (AEL)), **(B)** L2 (48-h AEL), and **(C)** L3 (96-h AEL) larvae. Testis hub is oriented towards the left. **(D-F)** 5-day-old wt testis stained for F-actin with phalloidin (green) and immunostained for Dlg (red). **(E)** and **(F)** are enlargements of **(D)**, displaying Dlg staining in the hub region and growing SCs under the surface of the testis, respectively. **(G-J)** wt testis stained for F-actin with phalloidin (green) and immunostained for Dlg and Eya (red) to visualize better the position of the SCCs. **(G** and **H)** Hub region, **(I** and **J)** growing spermatocyte cyst. **(H)** and **(J)** display only the Dlg and Eya staining of **(G)** and **(I)**, respectively. Arrows indicate the Eya positively stained nuclei of SCCs. Bar: 15 μm.

were closely linked to the periphery of germline cysts in association with the Dlg-stained cortical matrix of SCCs. The spatially restricted distribution at the distal edge of growing SCs suggests that Dlg may regulate cyst growth and differentiation.

dlg expression in somatic cells can restore development of dlg mutant testes

To determine more precisely the critical period of *dlg* function in the interaction between soma and germline, we tested first whether the JAK-STAT-signaling path-

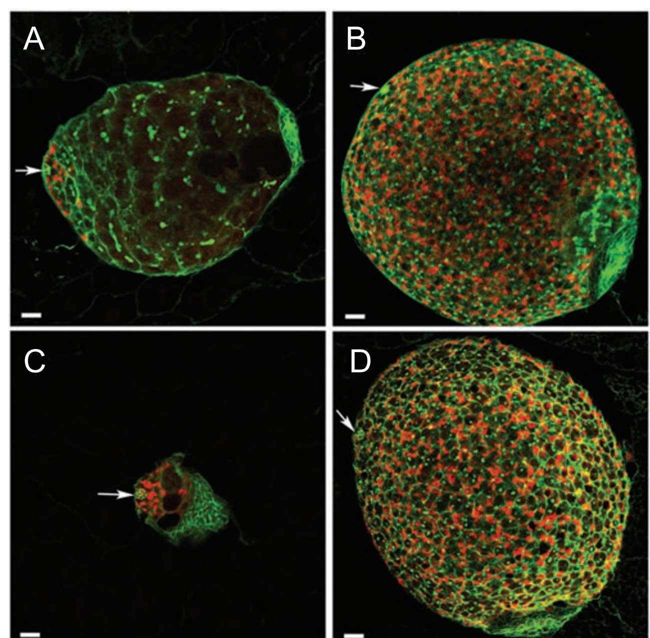

Figure 5 Unpaired drives stem cell proliferation in *dlg*. **(A)** wt, **(B)** *UAS-upd/+; nanos-Gal4*, **(C)** *dlg*, and **(D)** *dlg; UAS-upd/+; nanos-Gal4* L3 testes stained for Tj (red) and Arm+a-Spectrin (green). Ectopic *upd* expression in wt and *dlg* testes led to over-proliferation of spectrosome containing cells, indicating stem cell or gonialblast identity, and cells expressing the Tj marker, revealing a somatic identity. Arrows indicate the hub. Bar: 15 μm.

way was functional. The growth factor Upd is secreted by the hub cells and acts on GSCs to control their self-renewal [12, 13]. As shown in Figure 5 ectopic expression of *upd* in both wt and *dlg* prevented differentiation of gonialblasts and led to the accumulation of GSC- and gonialblast-like cells showing that the JAK-STAT pathway was apparently not affected in *dlg* testes. These data indicate that *dlg* is dispensable for the interaction between germline and somatic cells involving the JAK-STAT pathway.

To confirm that *dlg* plays a critical role in somatic cells during their differentiation, we specifically expressed a *UAS-dlg* transgene in either somatic or germ cells in *dlg* testes by using specific *Gal4* drivers [31]. Three drivers direct Dlg synthesis in somatic cells, including *c833* (Figure 6A and E), *c729* (Figure 6B and F), and *T155* (Figure 6C and G), whereas the *nanos* driver directs Dlg production in germ cells (Figure 6G and H). Combination of *UAS-dlg* with the somatic drivers in *dlg* mutant restored early testis development, whereas the combination with the germline driver was ineffective (Figure 6). The *c833* construct drives expression of reporter genes in the hub, SSCs, and early SCCs, and *c729* in SSCs, early SCCs, and testis sheath cells, whereas *T155* predominantly directs expression in late SCCs [32]. All three somatic drivers in combination with *UAS-dlg* led to the formation of SCs and spermatocyte cysts containing branched fusomes in germ cells and producing Eya in somatic cells. The overall size of the rescued testes was slightly smaller than that in wt. Expression of *dlg* driven by *c729* and *c833* produced spermatocyte cysts, which in the distal part of the testis showed a round structure instead of the scale shape characteristic of wt cysts. With *T155*, the larger cysts were even more distorted and their limits were poorly defined. Dlg synthesis could be detected in the rescued testes. With *c729* (Figure 6I-J), the

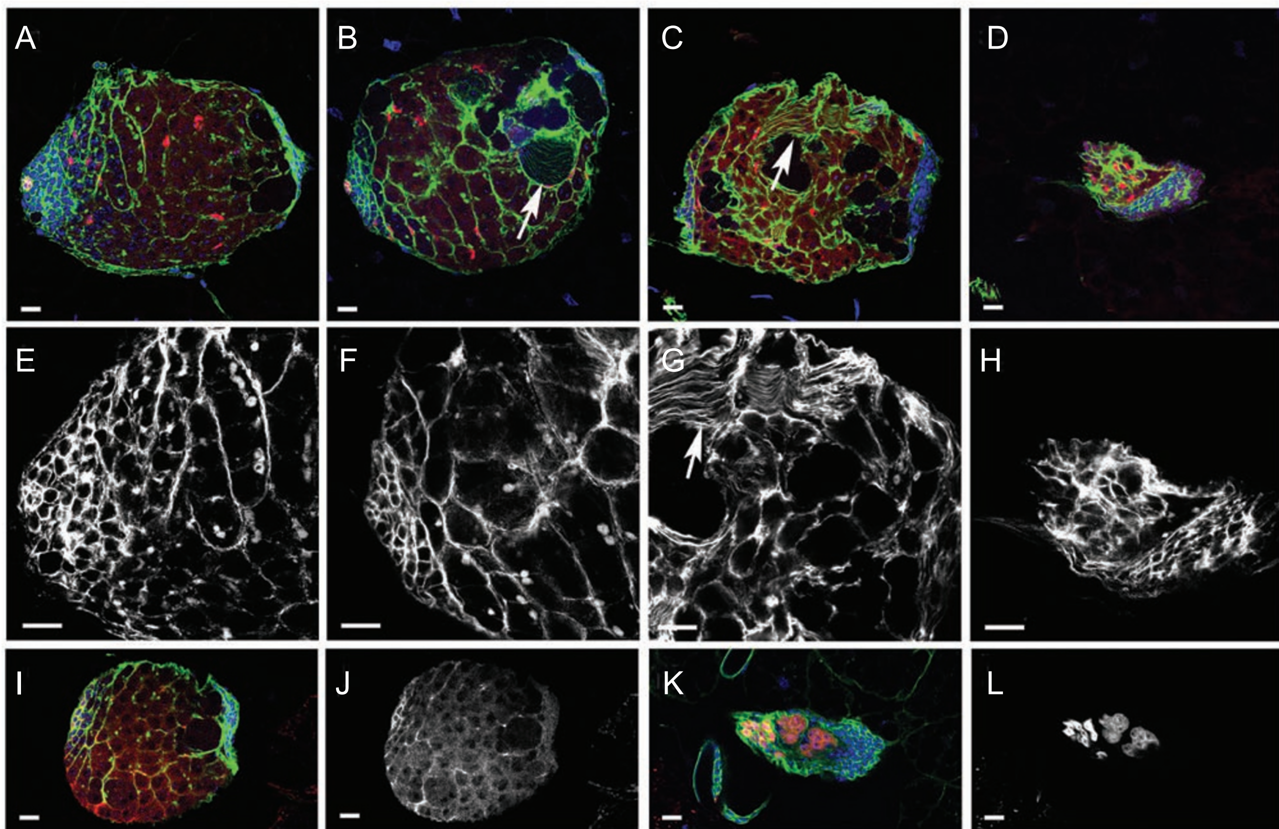

Figure 6 *dlg* expression in somatic cells restores testis development in mutant *dlg* larvae. Expression in *dlg* testes of *UAS-dlg* in somatic cells driven by **(A**, **E)** *c833-Gal4*, **(B**, **F**, **I**, **J)** *c729-Gal4*, **(C**, **G)** *T155-Gal4,* in germline cells (**D**, **H**, **K**, **L**) by *nanos-Gal4.* In **(A**-**D)**, DNA was visualized with DAPI (blue), F-actin with phalloidin (green), the hub with Fasciclin III (red), and SCCs wrapping spermatocyte cysts with Eya (red). **(E**-**H)** Enlargements of **(A**-**D)** showing F-actin distribution. In **I** to **L**, testes were stained for Dlg (red), DNA was visualized with DAPI (blue) and F-actin with phalloidin (green). **J** and **I** show only the Dlg staining of **I** and **K**, respectively. Testis hub is oriented towards the left. White arrows point out post-meiotic cysts with elongating spermatids. Bar: 15 μm.

Dlg staining was relatively diffused between the cysts, whereas Dlg synthesis was particularly pronounced in germline cells under the *nanos* driver (Figure 6K-L). The *c833* driver gave similar results as the c729, whereas the *T155* driver gave rise to a relatively intensive staining in late SCCs (data not shown). Furthermore, in 6- to 8-day-old transgenic larvae we occasionally found cysts of elongated spermatids, indicating that meiosis had taken place (Figure 6, arrows).

dlg-RNAi in SCCs blocks testis development

To further evaluate the developmental period during which *dlg* inactivation led to defective testis development, we silenced *dlg* expression in somatic cells by RNA interference. For this purpose, we depleted *dlg* transcripts by combining a *UAS-dlg-RNAi* transgene [33] with any of the three somatic drivers and examined the resulting phenotype. The combination of *c833* and *c729* with *UAS-dlg-RNAi* led to fully atrophied testes similar to those found in *dlg* mutant larvae (data not shown). In contrast, combination with *T155* had no major effect on the initial formation of SCs, which contained branched fusomes, but affected the structure of more advanced cysts (Figure 7). In these cysts, the germ cells were predominantly individualized with fragmented fusomes forming small nodes. When *dlg* inactivation was performed in the germ cells, no phenotype was detected (Supplementary information, Figure S1). Our data further indicated that *dlg* function was primarily required in early SCCs, whereas *dlg* inactivation in late SCCs led to the disruption of cyst organization.

dlg overexpression triggers cyst differentiation

In order to gain more insights into the development of cysts, we overexpressed *dlg* in wt testes by combining the *UAS-dlg* construct with either *c729* or *T155*. Both drivers produced a similar phenotype in L3 testes (Figure 8 and Supplementary information, Figure S2). These testes were particularly filled with large cysts, and the distal somatic cells contained a marked accumulation of Dlg, which formed a scalloped fringe instead of the smooth pattern seen in wt. All together our data indicate that *dlg* expression is particularly required in somatic cells at the beginning of cyst expansion to drive their differentiation.

Discussion

Our data demonstrate that *dlg* acts as a key regulator of testis morphogenesis and is primarily required in cells of somatic origin. In the *dlg* mutant testes, the SSCs and early SCCs survived as indicated by the positive Tj staining, whereas the late SCCs died, as revealed by the positive caspase-3 staining and the absence of Eya-staining. The critical period of *dlg* function takes place between the time of *tj* inactivation in somatic cells surrounding SCs [26] and that of *eya* activation in somatic cells wrapping spermatocyte cysts [27]. The shift between *tj* and *eya* expression coincides with a marked change in Dlg distribution. Dlg is first present at the interface of all cells forming the apex of the larval testis including the hub, GSCs, SSCs, and during early stages of SC differentiation becomes restricted to the somatic cells capping the growing spermatocyte cysts.

As mutations in *tj* encoding the *Drosophila* Maf transcription factor primarily prevent somatic cells to intermingle between germ cells [26], the occurrence in *dlg* testes of numerous Tj-positive cells mixed with germ cells indicates that *dlg* inactivation has no effect on this process. The *eya* gene encodes a transcriptional activator, which in combination with Sine oculis (So), forms

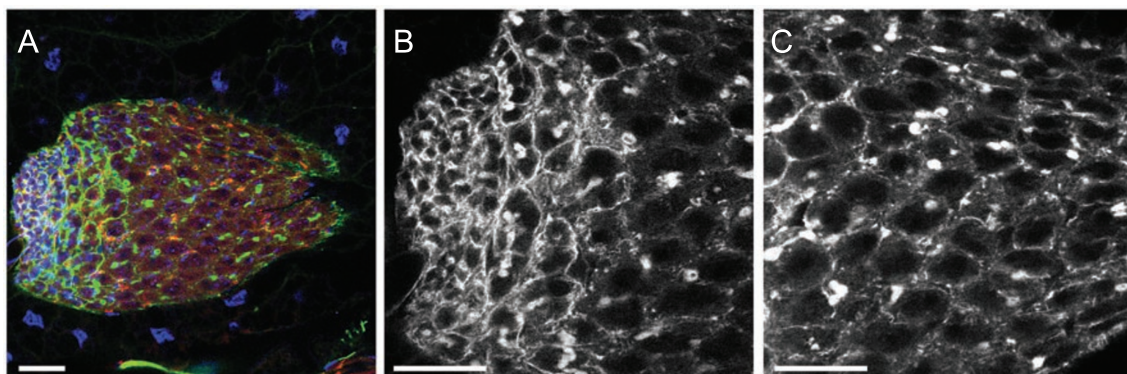

Figure 7 *dlg*-RNAi in SCCs blocks testis development. Depletion of *dlg* transcripts by *UAS-dlg-RNAi* driven by *T155-Gal4*. **(A)** View of a full transgenic L3 testis. The testis was stained for DNA (blue), F-actin (green), and Dlg (red). **(B** and **C)** Higher magnifications showing F-actin distribution in **(B)** apical and **(C)** distal regions of the testis shown in **(A)**. Bar: 15 µm.

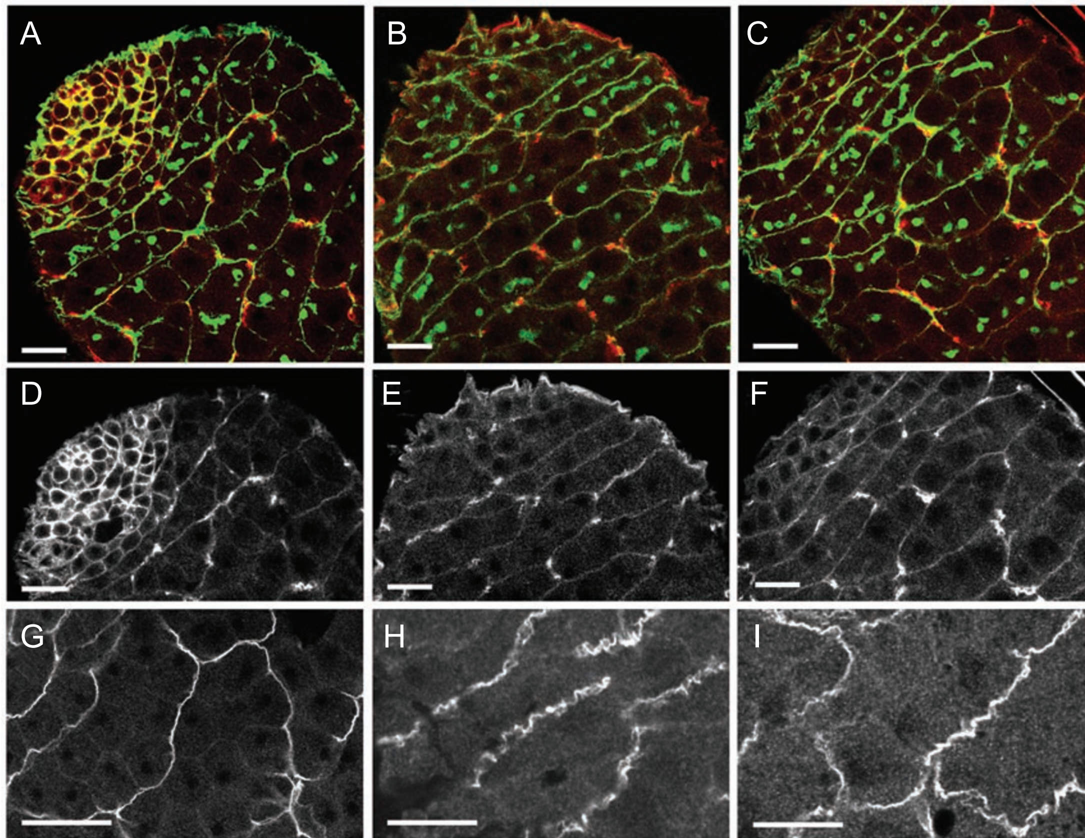

Figure 8 *dlg* overexpression triggers cyst differentiation. wt testis **(A)** and transgenic testes expressing *UAS-dlg* driven by **(B)** *c729-Gal4* and **(C)** *T155-Gal4.* Testes were stained for F-actin (green) and Dlg (red). **(D-F)** Dlg stained apical domains of testes shown in **(A-C)**. **(G-I)** Higher magnifications of Dlg distribution at the surface of spermatocyte cysts of testes shown in **(A-C)**. Bar: 15 µm.

a transcription complex required for early eye specification [34]. Clonal analysis reveals that both *eya* and *so* are required in late SCCs to ensure the survival of spermatocyte cysts during the relatively long phase of growth prior to meiosis, albeit not at the onset of spermatocyte growth [27]. Thus, *eya* inactivation leads to cell death in SCCs later than that produced by *dlg*. We suggest that the downregulation of *eya* observed in *dlg* testes is a secondary effect of the *dlg* mutation.

dlg expression driven by *c729* and *c833* in *dlg* mutant testes was able to restore normal development in the major part of the testes particularly around the hub and at the spermatogonial stages, whereas the pattern of spermatocyte cysts in the distal part of the testes was partially distorted. *dlg* expression driven by *T155,* which should predominantly occur in somatic cells surrounding spermatocyte cysts, was also able to restore development. However, the larger spermatocyte cysts were unable to aggregate in a compacted form, although some of the cysts were able to undergo meiosis. These findings demonstrate that germ cell development could be restored when *dlg* was expressed in somatic cells, presumably at different stages of their development.

Depletion of *dlg* transcripts by RNAi in SSCs and early SCCs led to the formation of fully degenerated gonads, whereas the same depletion performed in late SCCs with *T155* resulted in cyst fragmentation and an apparent individualization of the spermatocytes, albeit not germ cell death as observed in *dlg* testes. The contrasted effects produced by RNAi depletion between early and late periods of cyst differentiation further show that *dlg* plays two distinct functions during early testis development. The *T155* results indicate that *dlg* acts to regulate the growth and differentiation of spermatocyte cysts. Collectively, our data support the idea that first *dlg* acts in SSC to properly enclose growing SCs and establish functional junctions with germ cells, which are required for the survival of both germline and somatic cells. Then during a second period of testis development, *dlg* is needed to maintain the structural integrity of the growing cysts and

trigger polarized growth in an anterior-posterior direction. The concept of a *dlg* requirement in cyst-polarized growth is further supported by the finding that the amount of Dlg is markedly increased in capping cells of wt cysts overexpressing *dlg*. In addition, overexpression of *dlg* by the somatic drivers led to an increased number of differentiating cysts at the testis apex, suggesting that *dlg* acts on cyst differentiation.

Testes of *dlg* larvae displayed a comparatively smaller number of GSCs loosely attached to the hub. The reduced rate of GSC division in *dlg* appeared to be extrinsic and to result from the inability of SSCs to properly establish contact with GSCs, gonialblasts, and germ cells. The requirement of *dlg* in somatic tissues is further supported by the finding that GSCs survived following RNAi depletion of *dlg* transcripts in germ cells and, conversely, *dlg* expression in mutant GSCs was unable to restore their viability. Although a smaller number of GSCs were detected around the hub of *dlg* mutant testes, these cells apparently gave rise to asymmetric cell divisions generating gonialblasts, which in turn divided mitotically to form spermatogonial cells. This is in contrast to the deregulated asymmetric cell division occurring in *dlg* neuroblasts, which divide equally due to incorrect positioning of cell polarity determinants and generate neoplasms [6, 8, 35, 36].

Known defects in asymmetric cell division of male GSCs result from an aberrant positioning of centrosomes, leading to spindle misorientation [6-8]. Mutations in genes regulating microtubule polymerization, such as *centrosomin*, or encoding components anchoring astral microtubules to the hub-GSC interface such as *apc2,* a homolog to the human tumor suppressor adenomatous polyposis coli, moderately increase the number of GSCs, which, however, maintain direct contact with the hub [5]. However, overproliferation of germ cells can operate in gonialblasts and presumably two-cell SCs. The *bag-of-marbles* (*bam*) and *benign gonial cell neoplasm* (*bcgn*) genes act autonomously to restrict proliferation of amplifying germ cells, but not of stem cells [37]. Moreover, a recent report shows that Dlg is not among the genes that are up- or downregulated by JAK-STAT in a *bcgn* background [38], although the Dlg protein is robustly synthesized in the hub of wt testes. These data indicate that the mechanism leading to overproliferation of male germ cells is distinct from that in neuroblasts.

dlg overexpression in SCCs led to Dlg accumulation in cells capping the spermatocyte cysts. The region decorated by Dlg on the plasma membrane is clearly delineated and is spread over a limited domain of the cyst surface. The precise extension of somatic cells over germ cell cysts and Dlg distribution in these cells require more detailed studies. However, *dlg* overexpression had no apparent influence on the spreading of somatic cells over spermatocyte cysts. We observed only a thickening of the Dlg deposit, as well as a greater waving and ruffling of the cyst cells. The cell ruffling detected in somatic cells is highly reminiscent of the formation of lammellipodia-like structures upon upregulation of Rac1 in SCCs [17]. Rac1 is a downstream component of the Egfr pathway and acts antagonistically to Rho to regulate germ cell encapsulation. In the classical view of *Drosophila* Egfr pathway, the downstream kinase binds to the stimulated Egfr and activates a MAP-kinase cascade for transcription of target genes [39]. However, in *Drosophila* testis, the Egfr pathway is branched at the level of the docking protein so that binding of the adaptor protein Vav to Egfr activates the small GTPase Rac1 [17]. As the Dlg protein plays a central role in the organization of epithelial junctions and in signal transduction at sites of cell-cell contact, it is possible that the C-terminal tail of Egfr interacts with one of the PDZ domains of Dlg [40, 41]. In this way *dlg* inactivation would result in a disruption of the Egfr protein complexes at cellular junctions, block the Egfr pathway, and impair Rac1 function. Following *dlg* overexpression, we detected an excess of differentiating cysts at the testis apex at the expense of early SCs. Egfr upregulation can result in a similar effect [14, 15]. Based on these data, we hypothesize that Dlg may act on the cytoskeleton of the somatic cells to mediate cell-shape changes leading to either cellular extensions over the SCs, or reinforcing cell-to-cell contacts with growing germ cells.

Whether our findings may be extended to higher vertebrates remains an open question. Nevertheless, in mammals like in *Drosophila*, spermatogenesis depends on interactions between supportive Sertoli cells and germ cells [42]. Moreover, inactivation of the *Dlgh-1* gene in mice produces various abnormalities in their renal and urogenital organs with hypoplasia of kidneys and ureters, and absence of vagina and seminal vesicle [43, 44]. Generation of mice conditional for the expression of homologs of the *Drosophila dlg* would provide a suitable system for investigating their functions during mammalian gonadogenesis.

Material and Methods

Drosophila stocks

Oregon R was used as wt. The dlg^{m52} and *UAS-dlg* lines were obtained from Chris Doe, *UAS-upd* from Georg Halder, and dlg^{X12} from David Bilder. The *UAS-dlg-RNAi* lines were provided by the Vienna Drosophila RNAi Center (VDRC, Austria), whereas the *nanos-Gal4VP16*, *c833-Gal4*, *c729-Gal4*, *T155-Gal4*, and *UAS-p35* lines were obtained from the Bloomington Stock Center (Indi-

ana). Other fly stocks used in this study are described in FlyBase.

Immunofluorescence staining and microscopy

Testis immunostaining [22] was modified as follows: Testes were incubated overnight with primary antibodies in 1% PBX (1% Triton-100X in PBS) at 4 °C. Confocal images were obtained using a Leica system TCS SP2 and processed with Adobe Photoshop 7.0.

The following antisera were used: rat polyclonal anti-Traffic-jam (Tj) antibodies (1:2 000, gift from D Godt), rabbit polyclonal anti-Scrib1 antibodies [22] (1:1 000) and rabbit monoclonal caspase-3 antibody (1/1 000, Cell Signalling Technology, MA, USA). The mouse monoclonal anti-Dlg antibody was produced in hybridomal cells [45] (gift from C Doe). The mouse monoclonal anti-Armadillo N7A1 (1/10), anti-FasIII (1/100), anti-eya10H6 (1/100), anti-α-spectrin (3A9) (1/100) antibodies, and the rat monoclonal anti-Vasa (1/10) antibody were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. F-actin was stained with Alexa Fluor phalloidin 488 and 546 (1/300, Invitrogen, Karlsruhe, Germany) and DNA with DAPI (Invitrogen). Following secondary antibodies were used: Alexa Fluor 647 goat anti-guinea pig IgG (1/300, Invitrogen), Alexa Fluor 546 goat anti-rabbit IgG (1/300, Invitrogen), Cy5-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rat IgG (Jackson Immunochemistry, PA, USA).

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References

1 Santos AC, Lehmann R. Germ cell specification in *Drosophila* and beyond. *Curr Biol* 2004; **14**:R578-R589.
2 Casper A, Van Doren M. The control of sexual identity in the *Drosophila* germline. *Development* 2006; **133**:2783-2791.
3 Godt D, Tepass U. Organogenesis: keeping in touch with the germ cells. *Curr Biol* 2003; **13**:R683-R685.
4 Fuller MT, Spradling AC. Male and female *Drosophila* germline stem cells: two versions of immortality. *Science* 2007; **316**:402-404.
5 Yamashita YM, Jones DL, Fuller MT. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science* 2003; **301**:1547-1550.
6 Yamashita YM, Fuller MT. Asymmetric stem cell division and function of the niche in the *Drosophila* male germ line. *Int J Hematol* 2005; **82**:377-380.
7 Yamashita YM, Mahowald AP, Perlin JR, Fuller MT. Asymmetric inheritance of mother versus daughter centrosome in stem cell division. *Science* 2007; **315**:518-521.
8 Gonzalez C. Spindle orientation, asymmetric division and tumour suppression in *Drosophila* stem cells. *Nat Rev Genet* 2007; **8**:462-472.
9 Lin H. The stem-cell niche theory: lessons from flies. *Nat Rev Genet* 2002; **3**:931-940.
10 Gönczy P, DiNardo S. The germ line regulates somatic cyst cell proliferation and fate during *Drosophila* spermatogenesis. *Development* 1996; **122**:2437-2447.
11 Fuller MT. In: Bate M, Martinez Arias A, eds. The development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, 1993:71-147.
12 Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* 2001; **294**:2542-2545.
13 Tulina N, Matunis E. Control of stem cell self-renewal in *Drosophila* spermatogenesis by JAK-STAT signaling. *Science* 2001; **294**:2546-2549.
14 Kiger AA, White-Cooper H, Fuller MT. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature* 2000; **407**:750-754.
15 Tran J, Brenner TJ, DiNardo S. Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. *Nature* 2000; **407**:754-757.
16 Schulz C, Woods CG, Jones DL, Tazuke SI, Fuller MT. Signaling from germ cells mediated by *rhomboid* homolog *stet* organizes encapsulation by somatic support cells. *Development* 2002; **129**:4523-4543.
17 Sarkar A, Parikh N, Hearn SA, Fuller MT, Tazuke SI, Schulz C. Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment. *Curr Biol* 2007; **17**:1253-1258.
18 Matunis E, Tran J, Gönczy P, Caldwell K, DiNardo S. *punt* and *schnurri* regulate a somatically derived signal that restricts proliferation of committed progenitors in the germ line. *Development* 1997; **124**:4383-4391.
19 Woods DF, Bryant PJ. The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* 1991; **66**:451-464.
20 Woods DF, Hough C, Peel D, Callaini G, Bryant PJ. Dlg protein is required for junction structure, cell polarity, and proliferation control in *Drosophila* epithelia. *J Cell Biol* 1996; **134**:1469-1482.
21 Bilder D, Perrimon N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* 2000; **402**:676-680.
22 Li M, Marhold J, Gatos A, Török I, Mechler BM. Differential expression of two scribble isoforms during *Drosophila* embryogenesis, *Mech Dev* 2001; **108**:185-190.
23 Strand D, Raska I, Mechler BM. The *Drosophila* lethal(2) giant larvae tumor suppressor protein is a component of the cytoskeleton. *J Cell Biol* 1994; **127**:1345-1360.
24 Marhold J, Papagiannouli F, Li M, Patel A, Mechler BM. Requirements for *scribble* expression in newly formed gonads of *Drosophila* embryos. *Gene Expr Patterns* 2003; **3**:143-146.
25 Lin H, Yue L, Spradling AC. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* 1994; **120**:947-956.
26 Li MA, Alls JD, Avancini RM, Koo K, Godt D. The large Maf factor Traffic Jam controls gonad morphogenesis in *Drosophila*. *Nat Cell Biol* 2003; **5**:994-1000.
27 Fabrizio JJ, Boyle M, DiNardo S. A somatic role for *eyes absent (eya)* and *sine oculis (so)* in *Drosophila* spermatocyte

development. *Dev Biol* 2003; **258**:117-128.
28 Hempel LU, Oliver B. Sex-specific DoublesexM expression in subsets of *Drosophila* somatic gonad cells. *BMC Dev Biol* 2007; **7**:113.
29 Hay BA, Wolff T, Rubin GM. Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* 1994; **120**:2121-2129.
30 Huh JR, Guo M, Hay BA. Compensatory proliferation induced by cell death in the *Drosophila* wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* 2004; **14**:1262-1266.
31 Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1993; **118**:401-415.
32 Hrdlicka L, Gibson M, Kiger A, *et al.* Analysis of twenty-four lines in *Drosophila* melanogaster. *Genesis* 2002; **34**:51-57.
33 Dietzl G, Chen D, Schnorrer F, *et al.* A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 2007; **448**:151-156.
34 Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL. The eye-specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. *Cell* 1997; **91**:881-891.
35 Albertson R, Doe CQ. Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat Cell Biol* 2003; **5**:166-170.
36 Knoblich JA. Mechanisms of asymmetric stem cell division. *Cell* 2008; **132**:583-597.
37 Gönczy P, Matunis E, DiNardo S. *bag-of-marbles* and *benign gonial cell neoplasm* act in the germline to restrict proliferation during *Drosophila* spermatogenesis. *Development* 1997; **124**:4361-4371.
38 Terry NA, Tulina N, Matunis E, DiNardo S. Novel regulators revealed by profiling *Drosophila* testis stem cells within their niche. *Dev Biol* 2006; **294**:246-257.
39 Shilo BZ. Signaling by the *Drosophila* epidermal growth factor receptor pathway during development. *Exp Cell Res* 2003; **284**:140-149.
40 Humbert P, Russell S, Richardson H. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* 2003; **25**:542-553.
41 Funke L, Dakoji S, Bredt DS. Membrane-associated guanylate kinases regulate adhesion and plasticity at cell junctions. *Ann Rev Biochem* 2005; **74**:219-245.
42 Fink C, Weigel R, Hembes T, *et al.* Altered expression of ZO-1 and ZO-2 in Sertoli cells and loss of blood-testis barrier integrity in testicular carcinoma in situ. *Neoplasia* 2006; **8**:1019-1027.
43 Iizuka-Kogo A, Ishidao T, Akiyama T, Senda T. Abnormal development of urogenital organs in Dlgh1-deficient mice. *Development* 2007; **134**:1799-1807.
44 Mahoney ZX, Sammut B, Xavier RJ, *et al*. Discs-large homolog 1 regulates smooth muscle orientation in the mouse ureter. *Proc Natl Acad Sci USA* 2006; **103**:19872-19877.
45 Parnas D, Haghighi AP, Fetter RD, Kim SW, Goodmann CS. Regulation of postsynaptique structure and protein localization by the Rho-type guanine nucleotide factor dPix. *Neuron* 2001; **32**:415-424.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)