

The internal structure of embryonic gonads and testis development in *Drosophila melanogaster* requires *scrib*, *lgl* and *dlg* activity in the soma

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ABSTRACT Interest in the mechanism leading to the formation of the germline and its differentiation during *Drosophila* development, initiated even as soon as the first ever cloned tumour suppressor gene in *Drosophila*, the *lethal (2) giant larvae (lgl)*, had been identified. Further work has shown that the *lgl*, as well as *discs large-1 (dlg)* and *scribble (scrib)* tumor suppressor genes code for scaffolding proteins associated with either the cytoskeletal matrix or the septate junctions that act in common pathways in various tissues. This study analysed the role of *Dlg*, *Scrib* and *Lgl* in the embryonic gonads and testis of *Drosophila melanogaster*. Loss of *scrib*, *dlg* and *lgl* had no effect on gonad formation, but *Dlg* and *Scrib* in the gonadal mesoderm acted critically in the somatic wrapping of the pole cells and the internal structure of the *Drosophila* embryonic gonads. *Dlg* also affected the incorporation of the male-specific *Sox100B* positive mesodermal cells into the male embryonic gonads, yet *Sox100B* expression in *dlg* testis remained unaffected. Analysis at later stages revealed that *scrib* and *lgl* expression in the somatic lineage of the *Drosophila* testis, similar to what was previously shown for *dlg*, was indispensable for testis development and homeostasis, as depletion of these genes resulted in extensive testes defects. The data presented here emphasize the somatic requirement of *Scrib*, *Dlg* and *Lgl* in embryonic gonads, as well as in the *Drosophila* testis that underlines the importance of the somatic lineage in the establishment and maintenance of testis formation throughout successive developmental stages.

KEY WORDS: *dlg*, *lgl*, *scrib*, gonads, *Drosophila testis*

Introduction

Great interest on polarity genes initiated after observations that loss-of-function mutations in genes establishing and maintaining polarity in *Drosophila* lead to tumor formation with invasive properties, which subsequently placed *Drosophila* at the center of cancer analysis (Gateff, 1978). Application of molecular biology led to the isolation and characterization of the first tumor suppressor gene (TSG), the *lethal (2) giant larvae (lgl, l(2)gl or p127^{gl})* (Mechler *et al.*, 1985). *lgl* encodes a cytosolic protein which can bind to non-muscle myosin II and to the cytoskeletal matrix along the baso-lateral part of the epithelial plasma membrane (Strand *et al.*, 1994a, Strand *et al.*, 1994b). Likewise, mutations in *discs large-1 (dlg)* and *scribble (scrib)* genes cause also tissue overgrowth phenotypes. Their encoded proteins associate with septate junctions and function in the establishment and maintenance of cell polarity (Bilder and Perrimon, 2000, Li *et al.*, 2001, Woods *et al.*, 1996).

Both proteins contain multiple PDZ domains that can bind to the C-terminal tail of trans-membrane proteins, including membrane receptors, cytoskeletal and cytosolic proteins. As polarity scaffolds are nowadays considered to be dynamic organizing centers, that regulate site-specific protein targeting or exclusion from adjacent domains and provide the guiding cues for signaling molecules and targeted membrane insertion, studying these classical tumor suppressors in other tissue contexts, such as the germline, has gained new interest (Lecuit and Wieschaus, 2002, Papagiannouli and Mechler, 2010).

During the early embryonic life of *Drosophila melanogaster* the primordial germ cells, also known as pole cells, are set aside from the somatic lineage at the posterior pole of the embryo. The pole cells migrate through the embryo to reach the location of

Abbreviations used in this paper: CySC, somatic cyst stem cell; *dlg*, discs large; GSC, germline stem cell; *lgl*, lethal (2) giant larvae; SCC, somatic cyst cell; *scrib*, scribble.

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Accepted: 4 October 2012. Final, author-corrected PDF published online: 8th March 2013.

gonad formation and coalesce together with the somatic gonadal mesoderm to form the embryonic gonads (Santos and Lehmann, 2004). Then, the cells of the somatic gonadal mesoderm extend cellular projections among the germ cells and the gonad becomes compact and spherical (Casper and Van Doren, 2006, Van Doren *et al.*, 2003). The development of male and female gonads already differs at the time of gonad coalescence, as male-specific somatic gonadal cells join the posterior of the male gonad, albeit die by apoptosis in females (Casper and Van Doren, 2006, DeFalco *et al.*, 2003). The hub, formed at the anterior of the late embryonic male gonad, 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 the germline stem cells (GSCs) in the adult testis (Casper and Van Doren, 2006, Fuller and Spradling, 2007). 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 (Casper and Van Doren, 2006, Godt and Tepass, 2003). In the niche, the GSCs, each enclosed in a pair of somatic cyst stem cells (CySCs), are physically attached to the hub cells via adherens junctions (Fuller and Spradling, 2007). Upon asymmetric stem cell division, each GSC produces a new GSC attached to the hub and a distally located gonialblast, whereas

each CySC pair divides to generate two CySCs and two somatic cyst cells (SCCs) (Fuller and Spradling, 2007, Wong *et al.*, 2005, Yamashita *et al.*, 2005, Yamashita *et al.*, 2003). The gonialblast divides mitotically four more times to give rise to 16 interconnected spermatogonial cells, forming a cyst surrounded by the two SCCs. The SCCs grow without division, become flat, elongate, and form a thin layer around the spermatogonial cyst (Fuller, 1993).

The importance of the somatic lineage in establishing the architecture and in shaping the microenvironment of the *Drosophila* testis has been described in several cases. The role of the JAK-STAT and BMP signaling pathways, which are the major stem cell maintenance pathways in the male germline (Issigonis *et al.*, 2009, Kawase *et al.*, 2004, Kiger *et al.*, 2001, Tulina and Matunis, 2001, Zheng *et al.*, 2011), the role of the EGFR pathway promoting differentiation (Kiger *et al.*, 2000, Tran *et al.*, 2000) and the antagonistic roles of Rho and Rac in organizing the germ cell microenvironment (Sarkar *et al.*, 2007), as well as of the gap junction protein Zero population growth (Zpg) (Tazuke *et al.*, 2002) are examples that emphasize the role of soma-germline communication.

Previous work has shown that *scrib* encodes two proteins, Scrib1 and Scrib2, resulting from the alternative splicing of the primary *scrib* transcript (Li *et al.*, 2001). Scrib1 (onwards referred to as Scrib), which is required for the acquisition and maintenance of cell polarity in follicular epithelia (Bilder and Perrimon, 2000), is abundantly synthesized in the newly formed gonads and more particularly in the center of the gonads where the pole cells aggregate (Li *et al.*, 2001). Analysis of Scrib dynamics revealed a homogeneous Scrib distribution at the periphery of the pole cells in newly formed gonads. At a later stage, along with gonad compaction, Scrib forms a polygonal network around the pole cells and decorates also the interstitial spaces between the pole cells, suggesting that it is present in both the pole cells and the gonadal mesodermal cells (Marhold *et al.*, 2003). Scrib localization in the gonadal mesoderm is cell autonomous, as analysis of agametic gonads and pseudo-gonads made of aggregated pole cells revealed that Scrib production in the pole cells requires a direct contact to the gonadal mesoderm (Marhold *et al.*, 2003).

Additional work revealed a new role for the septate junction protein Dlg (Albertson and Doe, 2003, Hough *et al.*, 1997, Humbert *et al.*, 2003, Woods *et al.*, 1996) in the *Drosophila* testis (Papagiannouli and Mechler, 2009, Papagiannouli and Mechler, 2010). In contrast to the overgrowth phenotypes observed in imaginal discs and brain hemispheres, *dlg* inactivation leads to testis degeneration during early larval development. The *dlg* testes are extremely small, with reduced number of GSCs loosely attached to the hub. Dlg underlies the membrane of the hub cells, CySCs and SCCs, and *dlg* expression in these cells can rescue the mutant phenotype whereas expression in the germline cannot (Papagiannouli and Mechler, 2009). Dlg localization in CySCs establishes a tight connection between GSCs and CySCs and thereby preserves the niche architecture. In spermatogonial and spermatocyte cysts, *dlg* expression in late SCCs is critical for their survival, growth, expansion and for maintaining the cysts' microenvironment and integrity (Papagiannouli and Mechler, 2009).

These observations provide strong evidence for a role of all three TSGs in gonad establishment and testis formation in *Drosophila melanogaster*. By directly comparing the localization and function of *scrib*, *dlg* and *lgl* in embryonic gonads and testis, I was able to show that somatic requirement of Scrib, Dlg and Lgl is essential for

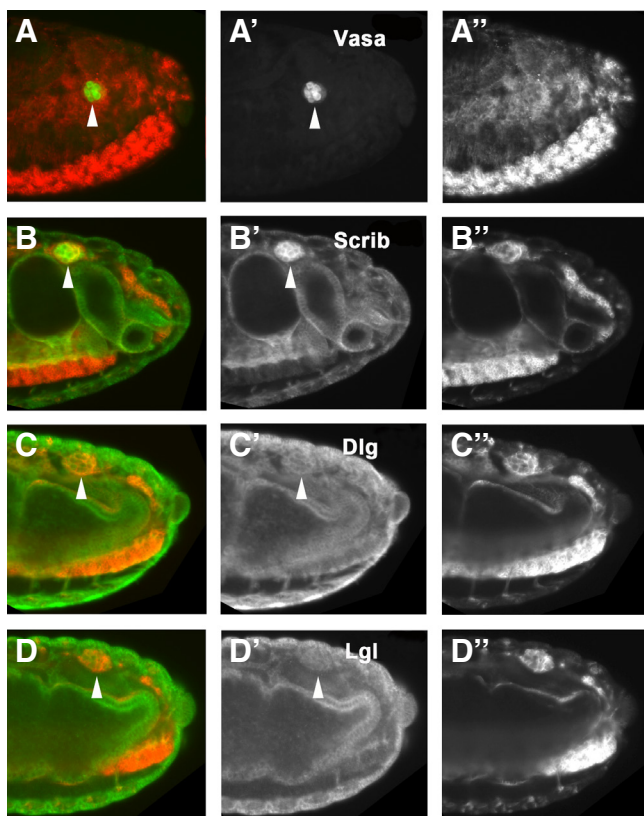


Fig. 1. Scrib, Dlg and Lgl localization in the newly formed gonads of *Drosophila* embryos. Parasagittal optical sections of whole-mount wild type (*wt*) embryos, stage 15-16, double-stained for Neurotactin (red) and Vasa (green) (A), Scrib (green) and Neurotactin (red) (B), Dlg (green) and Neurotactin (red) (C), and Lgl (green) and Neurotactin (red) (D). (A'-D'') show only the Neurotactin staining from (A-D), respectively. (A') Shows only the Vasa, (B') only the Scrib, (C') only the Dlg and (D') only the Lgl staining of (A), (B), (C) and (D), respectively. White arrowheads point at the gonads. All embryos are oriented dorsal up, posterior to the right.

TABLE 1

NUMBER OF MISPLACED POLE CELLS (MPC) PER EMBRYO IN WILD TYPE (WT), *LGL*, *SCRIB* AND *DLG* *DROSOPHILA* EMBRYOS OF STAGE 15-16

Genotype	Number of embryos	Misplaced pole cells (mpc)	mpc/embryo
wt	27	5	0,2
<i>lgl</i>	25	5	0,2
<i>scrib</i>	32	43	1,3
<i>dlg</i>	45	175	3,9

TABLE 2

NUMBER OF POLE CELLS (PC) WITHIN THE GONADS OF WILD TYPE (WT) AND *DLG* *DROSOPHILA* EMBRYOS OF STAGE 15-16

Genotype	Number of embryos	Total number of pole cells (pc)	pc/gonad
wt	48	584	12
<i>dlg</i>	35	393	11

establishing the gonadal architecture and for testis differentiation. Yet, the differences in the phenotypes observed among the three TSGs in embryonic and larval stages, suggest that the degree of their cooperation and interdependence can vary significantly according to the developmental stage.

Results

***Scrib*, *Dlg* and *Lgl* in *Drosophila* embryonic gonads**

In order to study the function of *scrib*, *dlg* and *lgl* in the embryonic *Drosophila* gonads, the distribution of the proteins encoded by these genes was determined (Fig. 1). Newly formed gonads of wild type (wt) stage 15 *Drosophila* embryos, were immunostained with Scrib (Fig. 1 B,B'), Dlg (Fig. 1 C,C') and Lgl (Fig. 1 D,D') specific antibodies. Neurotactin (Fig. 1 A-D and 1 A''-D'') was used in all cases as a marker for the gonadal mesoderm (de la Escalera *et al.*, 1990, Hortsch *et al.*, 1990, Marhold *et al.*, 2003) that encloses the pole cells of the newly formed gonads. Pole cells were also positively stained for Vasa (Fig. 1A,A'). This analysis revealed the presence of Dlg and Lgl in the embryonic *Drosophila* gonads, in levels similar to those of the surrounding tissue. Scrib showed a more intensive distribution in the gonads and predominant compared to the adjacent tissues.

Previous analysis of Scrib distribution in the embryonic gonads indicated that the gonadal mesoderm is required for Scrib synthesis in pole cells of the newly formed gonads (Marhold *et al.*, 2003). This finding prompted me to examine whether mutations in *scrib*, *dlg* and *lgl* embryos would alter pole cell migration and gonad formation. Examination of

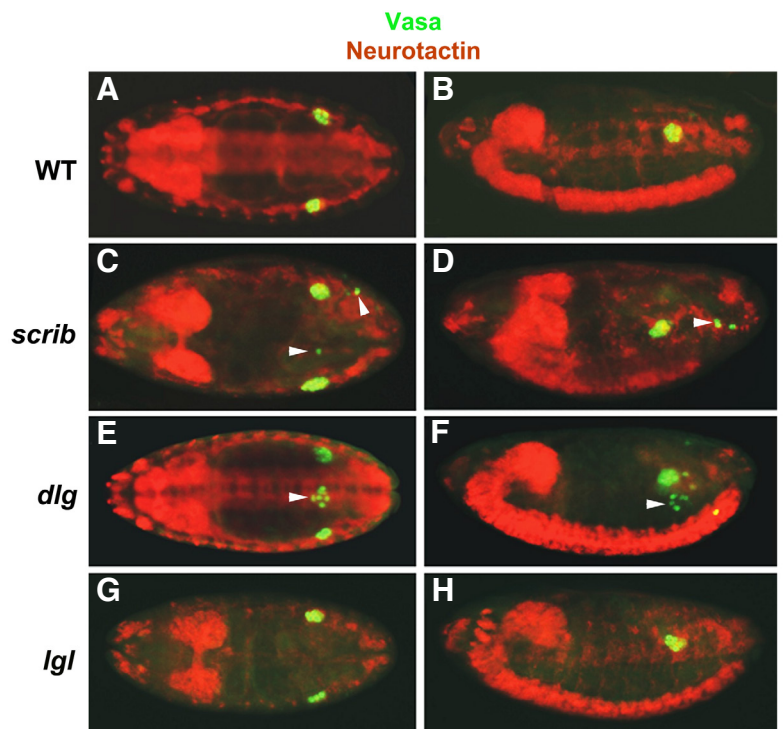
Fig. 2. Analysis of misplaced pole cells in *scrib*, *dlg* and *lgl* mutant embryos. Staining of wt (A,B), *scrib* (C,D), *dlg* (E,F) and *lgl* (G,H) mutant embryos of stage 15, with antibodies against Neurotactin (red) for the gonadal mesoderm, and Vasa (green) for the pole cells. (A,C,E,G) represent horizontal and (B, D, F, H) represent parasagittal optical sections. All embryos are oriented dorsal up, posterior to the right. Misplaced pole cells outside the newly formed gonads (white arrowheads) are observed in *scrib* (C,D) and *dlg* (E,F) mutant embryos.

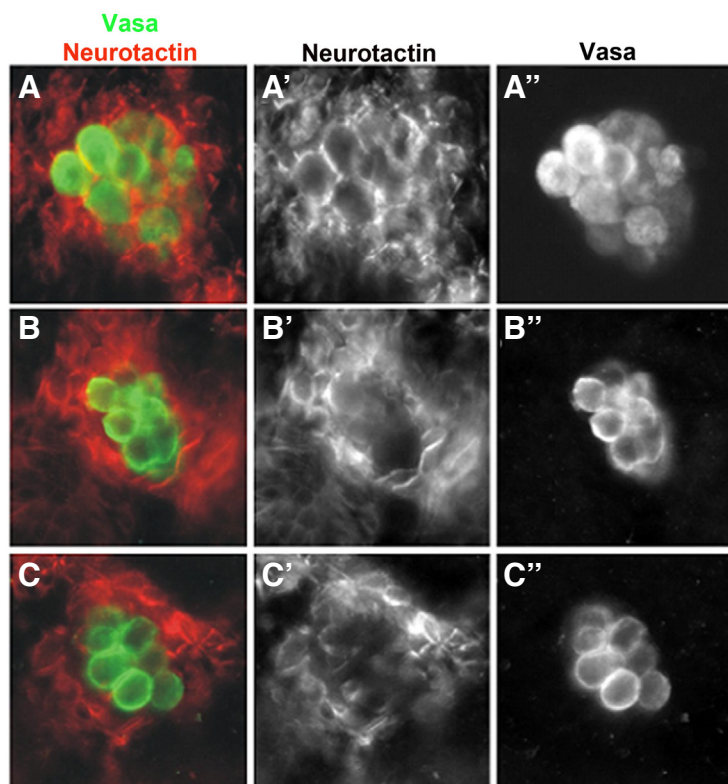
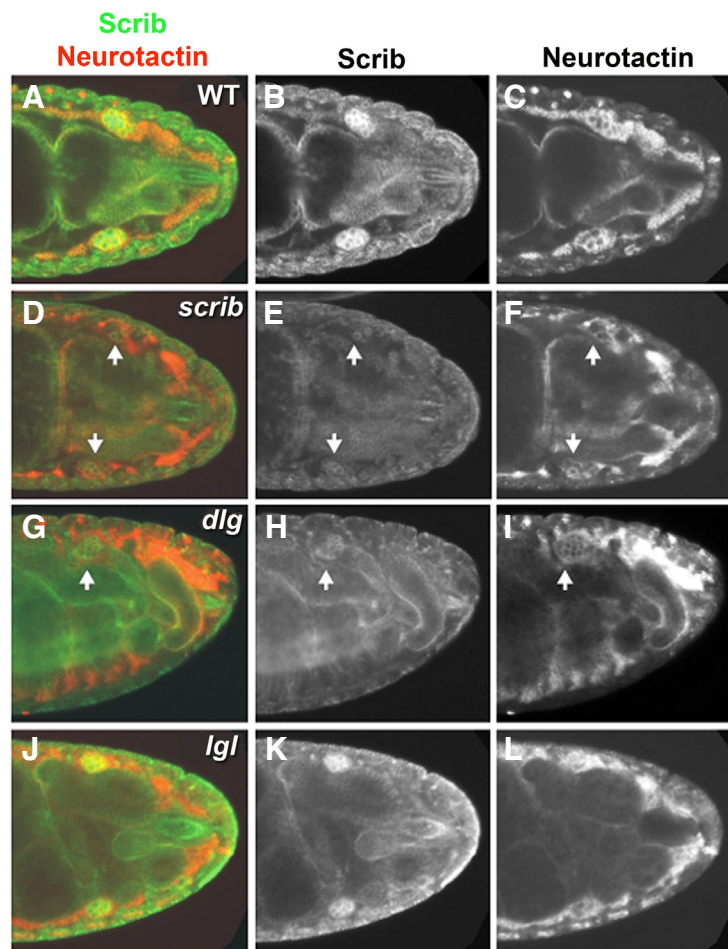
TABLE 3

NUMBER OF POLE CELLS (PC) PER GONAD IN RELATION TO MISPLACED POLE CELLS (MPC) IN *DLG* *DROSOPHILA* EMBRYOS OF STAGE 15-16

Misplaced pole cells	Total number of pole cells	Number of embryos	pc/gonad
0	68 (6+16+12+11+14+9)	6	11,13
1	68 (9+12+7+12+13+15)	6	11,13
2	108 (11+8+9+8+5+12+14+10+13+18)	10	9,8
3	111 (9+10+14+8+7+17+7+11+16+12)	10	10,1
4	38 (18+12+8)	3	12
Total number	393	35	11

homozygous mutants immunostained for Vasa and Neurotactin showed that gonads were formed in all three mutant embryos. Comparable analysis of late *scrib*, *dlg* and *lgl* homozygous mutant embryos (stage 14-16) revealed that during gonad assembly a significant number of pole cells was unable to reach the gonadal pockets in *scrib* and *dlg* embryos (Fig. 2; Table 1). Nevertheless, in *dlg* embryos showing the most severe pole cell misplacement phenotype, the number of pole cells forming the *dlg* gonads remained constant in comparison to the wt gonads (Table 2). Moreover, the number of pole cells in *dlg* gonads remained stable regardless of the number of misplaced pole cells in the same embryos (Table 3). Conclusively, the occurrence of misplaced pole cells in *dlg* embryos cannot be attributed to a failure in the migration process. One possible explanation would be that the misplaced pole cells correspond to pole cells that survive for a longer period of time in the mutants, similar to what was previously shown for *scattershot* and *outsiders* mutants (Coffman *et al.*, 2002).





Scrib is absent in gonads of *dlg* mutant embryos

Previous work in epithelial cells, neuroblasts and neuromuscular junctions (NMJ) has shown that Dlg and Scrib act together in a common pathway to regulate polarity. To check whether *scrib*, *dlg* and *lgl* depend on each other during gonad formation and early gonad development, wt, *scrib*, *dlg* and *lgl* mutant embryos (stage 15-16) were double stained for Scrib and Neurotactin (Fig. 3). This analysis revealed a normal Scrib distribution in wt (Fig. 3 A-C) and *lgl* gonads (Fig. 3 J-L). However, Scrib localization was lost not only in *scrib* gonads as expected (Fig. 3 D-F) but also in *dlg* mutant gonads (Fig. 3 G-I). Since the *scrib* gene function is not affected in *dlg* mutants, this finding suggested that Scrib requires Dlg for its proper localization, similar to what was shown previously for the neuroblasts and NMJ (Betschinger *et al.*, 2006, Caussinus and Gonzalez, 2005, Lee *et al.*, 2006, Roche *et al.*, 2002). The reciprocal staining showed that Dlg localized normally in *scrib* gonads (data not shown). These results correlate nicely with previous findings showing that Scrib is lost from the hub cells of *dlg* depleted larval testis (Papagiannouli and Mechler, 2009).

Absence of somatic wrapping affects the internal structure of the embryonic *scrib* and *dlg* gonads

The results obtained so far have clearly shown that gonad formation is not affected in any of the genes examined. Yet, the fact that the gonadal mesoderm is critical for Scrib localization in embryonic gonads (Marhold *et al.*, 2003) and that Dlg is required exclusively in the somatic lineage in the larval testis (Papagiannouli and Mechler, 2009), urged me to take a closer look at the internal structure and architecture of *scrib* and *dlg* depleted gonads. Staining of wt, *scrib* and *dlg* embryonic gonads with Vasa (Fig. 4 A''-C'') and Neurotactin (Fig. 4 A'-C'), to visualize the germ cells and gonadal mesoderm respectively, revealed an overall lower level and altered distribution of Neurotactin in both *scrib* (Figure 4B-B'') and *dlg* gonads (Fig. 4C-C''). In particular, the strong reticulated pattern of Neurotactin detected in between the pole cells of wt gonads (Fig. 4 A-A''), was absent in *scrib* and *dlg* gonads, suggesting a defect in the organisation of the mesodermal cells that normally extend cellular projections between the germ cells (Van Doren *et al.*, 2003). Therefore, mutations in *dlg* and *scrib* may exert no major effect on the coalescence of germ cells and gonadal mesoderm but affect the internal structure of the gonads.

Mesodermal male-specific Sox100B-cells fail to associate with the embryonic *dlg* male gonad

The defects detected in the gonadal mesoderm and internal

Fig. 3 (top). Scrib distribution in wild type, *scrib*, *dlg* and *lgl* gonads. Confocal images of wt (A-C), *scrib* (D-F), *dlg* (G-I) and *lgl* (J-L) late embryos (stage 16) stained for Scrib (green) and Neurotactin (red). Scrib staining is intense in the gonads of wt and *lgl* mutant embryos and is absent in *scrib* and *dlg* gonads. White arrows point at the gonads. All embryos are oriented posterior to the right.

Fig. 4 (bottom). Absence of somatic wrapping at the center of embryonic *scrib* and *dlg* gonads. Confocal images of embryonic gonads from late wt (A-A''), *scrib* (B-B'') and *dlg* (C-C'') embryos stained for Neurotactin (red) (A', B', C') and Vasa (green) (A'', B'', C''). The reticulated Neurotactin staining detected between the pole cells of the wt embryonic gonads is absent in the gonads of *scrib* and *dlg* mutant embryos.

structure of the *dlg* gonads raised the question of whether the male-specific gonadal mesoderm (DeFalco *et al.*, 2003, Nanda *et al.*, 2009) is also affected in *dlg* gonads. This cluster of male-specific mesodermal cells can be detected in both male and female stage 14 embryos as a group of cells located near the posterior end of the forming gonads (Hui Yong Loh and Russell, 2000), which becomes integrated only at the posterior part of the male and not of the female gonads (Casper and Van Doren, 2006). These cells express Sox100B (DeFalco *et al.*, 2003, Nanda *et al.*, 2009), the homologue of the Sox9 protein in mice that plays a critical role in sex determination and testis development (Clarkson and Harley, 2002, Cossais *et al.*, 2010). In order to address the role of *dlg* in the male-specific mesodermal cells, the fate of the Sox100B cells in *dlg* male gonads was analyzed. Double staining for Vasa and Sox100B of stage 14-17 wt male embryos (Fig. 5 A-B) showed that Sox100B-expressing cells cap the posterior end of embryonic gonads. These cells become associated with the male gonads (stage 14) even before the full compaction of the gonad (Fig. 5A) and can be still detected at this position during later embryonic stages 15-17 (Fig. 5B). As expected, no Sox100B-expressing cells become associated with the female gonads of 15-17 stage embryos (Fig. 5 C-D). However, examination of stage 15-17 *dlg* mutant embryos showed also an absence of Sox100B cells at the posterior end of the embryonic testes (Fig. 5 E-F). The lack of the Sox100B male-specific cells in *dlg* male gonads indicates that either *dlg* expression is required for the formation of the mesodermal Sox100B cells or that these cells can be formed and produce SoxB100, but become unable to adhere to the male gonad and are thereby lost.

To determine whether the mesodermal Sox100B cells were indeed formed in *dlg* embryos, *dlg* embryos at stage 14 were stained for Sox100B and Dlg, to readily identify mutant *dlg* embryos by their low content of the Dlg protein (Fig. 5 G-I). The perdurance of low amounts of the truncated Dlg^{m52} product (lacking the PDZ3 and SH3 domains) (Goode and Perrimon, 1997) was sufficient to assign the location of the male gonad. A group of 12-15 mesodermal cells positively stained for Sox100B could be detected in association with the posterior end of the male gonad in late stage 14 mutant *dlg* embryos (Fig. 5H). However, at stage 15 (Fig. 5I) no positively Sox100B-stained cells were detected in association with the *dlg* male gonad anymore. This suggests that in *dlg* male gonads, like in the case of female gonads, the Sox100B-cells can transiently become associated with embryonic gonads but fail to colonize the compact gonads of late embryonic stages. The conclusion, therefore, is that Dlg is critical for establishing the architecture in the newly formed gonad, involving the intimate contacts of germ cells and the gonadal mesoderm as well as the incorporation of the male-specific Sox100B cells in the embryonic male gonad.

Later requirement of Sox100B in the larval testis is not affected in *dlg* and *scrib* mutants

Sox100B is also expressed in another male-specific somatic gonad lineage, the pigment precursor cells, which are the embryonic precursors of the pigment cells (DeFalco *et al.*, 2008) forming the outer layer of the larval and adult testis responsible for the yellow colour of testis sheath and seminal vesicle (Fuller, 1993). It was therefore of particular interest to investigate whether Sox100B expression is affected in *dlg* mutant testes. For this purpose, wild type testes and ovaries, as well as mutant *dlg* and *scrib* testes,

were stained for SoxB100 and filamentous actin (F-actin). In all wt and mutant testes a strong SoxB100 signal in the cells of the outer sheath layer was observed, in particular in their nuclei, and no staining in the somatic cells located at the posterior end of wild type and mutant testes was detected (Fig. 5 J-L). Examination of ovaries revealed that the cells of the outer sheath layer exhibited also a strong positive signal for Sox100B (data not shown). In addition, the density of nuclei and thus of the cells forming the outer sheath layer was greater at the anterior of the testes than at the posterior. The data presented here, in accordance with previous

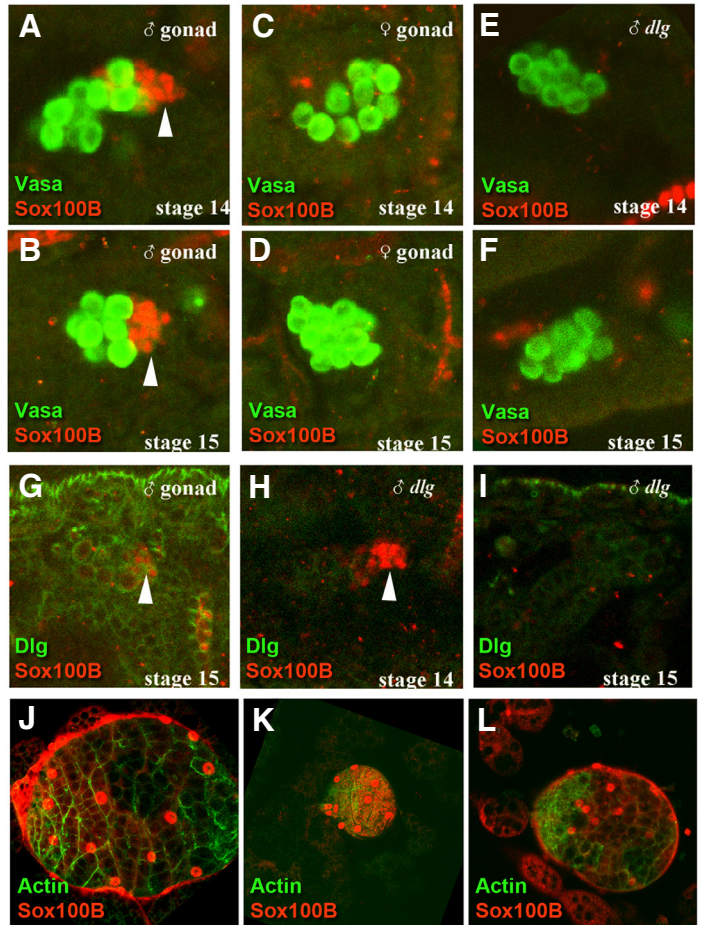


Fig. 5. Male-specific Sox100B cells are not incorporated in *dlg* embryonic male gonads. (A-F) Absence of Sox100B cells in *dlg* male gonads. Confocal images of wt male (A,B), wt female (C,D) and *dlg* embryos (E,F) of stage 14-15 double stained for Sox100B (red) and Vasa (green). Staining reveals that Sox100B expressing mesodermal cells cap the posterior end of embryonic testes but are absent from embryonic ovaries. (G-I) Sox100B cells are present in *dlg* embryos but fail to colonize the newly formed gonads. Confocal images of wt male stage 15 (G) and *dlg* embryos on stage 14 (H) and stage 15 (I), double stained for Sox100B (red) and Dlg (green). The staining reveals that the Sox100B cells are present in *dlg* embryos but fail to become incorporated in the posterior part of the forming testis. In (H), pole cells are not clearly visible since they are not at the same focal plane as the Sox100B cells. White arrowheads point at the Sox100B positive cells. All embryos are orientated anterior to the left. (J-L) Pattern of Sox100B localization in wt, *dlg* and *scrib* 3rd instar larvae testes. Confocal images of wt (J), *dlg* (K) and *scrib* (L) testes, double stained for Sox100B (red) and F-actin (green). Sox100B cells are found in the outer sheath layer of larval testis. Hub is orientated to the left.

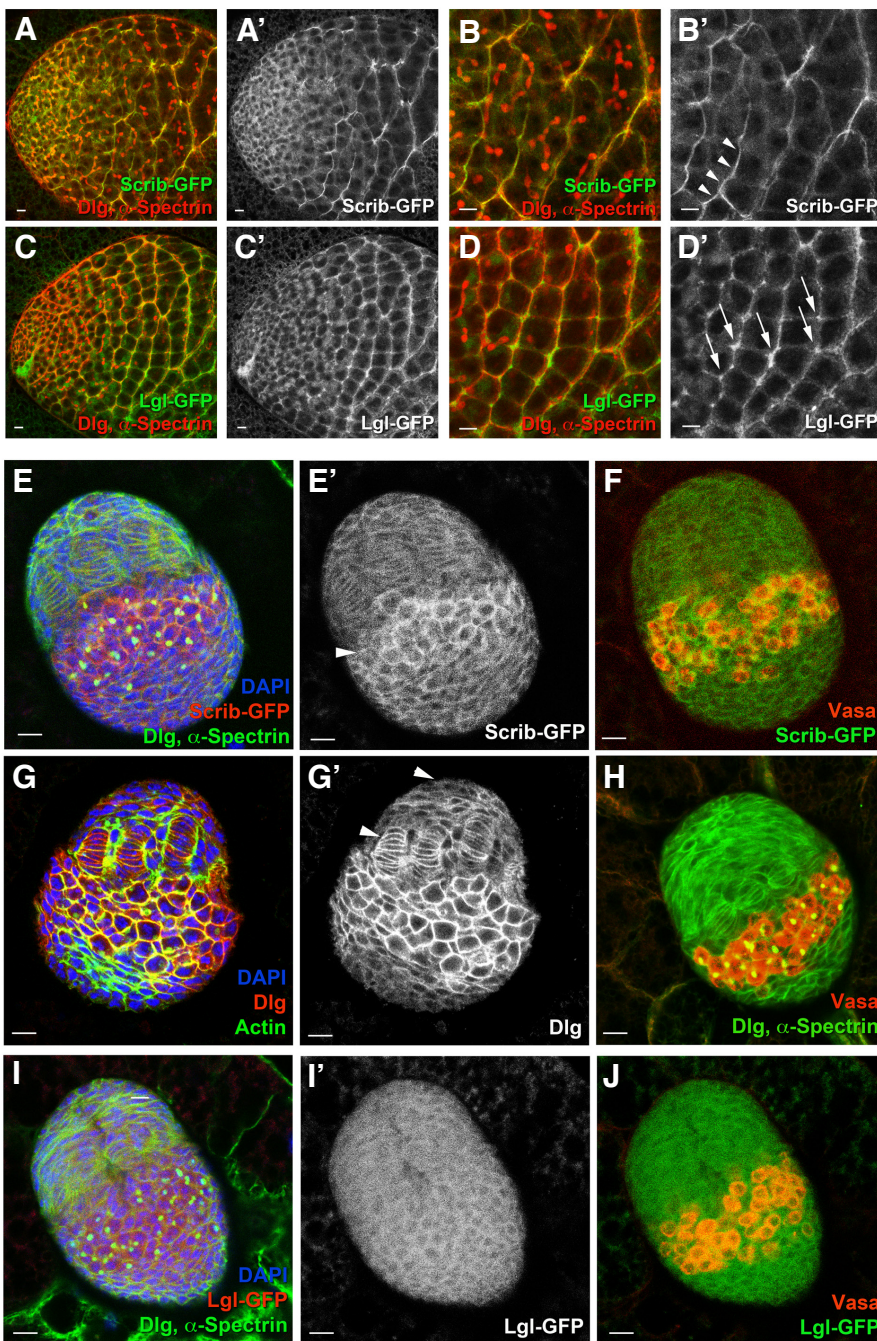


Fig. 6. Pattern of Scrib, Lgl and Dlg distribution in 3rd instar larval testes and ovaries. Stained testes (A-B') of a Scrib-GFP enhancer trap line showing Scrib localization (green) and (C-D') of an Lgl-GFP enhancer trap line showing Lgl localization (green), together with Dlg+ α -Spectrin specific antibodies (red). (B-B') and (D-D') are enlargements of (A-A') and (C-C') respectively, displaying the spermatocyte cysts. (A', B') display only the Scrib-GFP and (C', D') display only the Lgl-GFP, marking the hub, CySCs and SCCs. White arrowheads in (B') point at the Scrib smooth localization at SCCs. White arrows in (D') point at the Lgl accumulation at junction points. (E-F) Stained ovaries of a Scrib-GFP enhancer trap line (green) for Dlg+ α -Spectrin (red) and DAPI (blue) (E), or for Vasa (red) (F). (G-H) Stained wt ovaries for Dlg (red), F-actin (green) and DAPI (blue) (G), or for Dlg+ α -Spectrin (green) and Vasa (red) (H). (I-J) Stained ovaries of an Lgl-GFP enhancer trap line (green) for Dlg+ α -Spectrin (red) and DAPI (blue) (I), or for Vasa (red) (J). (E') shows only the Scrib-GFP of (E) and white arrowhead points at the intensive Scrib localization in the mesodermal cells surrounding the germline. (G') shows only the Dlg+ α -Spectrin staining of (G) and white arrowheads point at terminal and anterior cells. (I') shows only the Lgl-GFP of (I) depicting the weak Lgl localization in 3rd instar ovaries. Testis hub is oriented towards the left (white arrows). Scale bar, 15 μ m.

polarity genes in testis development.

Analysis of Scrib and Lgl localization in larval testis (Fig. 6 A-D') was done using *Scrib-GFP* and *Lgl-GFP* enhancer trap lines (Buszczak *et al.*, 2007, Kelso *et al.*, 2004). Co-staining with a Dlg specific antibody to visualize the somatic lineage and α -Spectrin antibody for the spectrosome and the fusome, showed that both Scrib and Lgl co-localize with Dlg in the hub, CySCs and SCCs (Fig. 6 A-D'). In particular, Scrib showed a uniform smooth distribution in the plasma membrane of CySCs and SCCs with short indentations at contact sites with neighboring germ cells (Fig. 6 A-B'). Lgl was associated with the plasma membrane of CySCs and SCCs but showed clearly an intensive accumulation at junction points with the adjacent germ cells (Fig. 6 C-D'). Similar results were observed by comparing Scrib, Dlg and Lgl distribution in the 3rd instar larval ovaries (Fig. 6 E-J). All three proteins localized in all somatic populations, with Scrib showing a marked distribution in the mesodermal cells surrounding the germ cells (Fig. 6 E-F), Dlg more intensively localized in the terminal filaments and anterior somatic cells (Fig. 6 G-H) whereas Lgl had an overall uniform somatic distribution (Fig. 6 I-J).

observations (Nanda *et al.*, 2009), suggest that the larval Sox100B-expressing cells constitute a population distinct from the embryonic Sox100B-positive cells detected in the male gonads.

***scrib* and *lgl* are required for testis differentiation**

In the embryonic *Drosophila* gonads, somatic requirement of Scrib and Dlg affects the internal structure of the gonads whereas Dlg acts critically on the incorporation of the male-specific somatic gonadal cells in the male gonads. These observations, together with previous results showing that *dlg* is required in the somatic population of the *Drosophila* testis to promote differentiation, survival and maintenance of testis architecture (Papagiannouli and Mechler, 2009), raised the possibility for a role of the apical-basal

observations (Nanda *et al.*, 2009), suggest that the larval Sox100B-expressing cells constitute a population distinct from the embryonic Sox100B-positive cells detected in the male gonads.

In wild type *Drosophila*, larval life consists of three successive larval stages, with a duration of one day for the 1st and 2nd larval stage (for flies raised at 25°C) and a 3rd instar larval stage of 2-3 days long (from 72 to 120 hours After Egg Laying), making a total of 5 days after egg laying before pupariation occurs. However, all three *dlg*, *scrib* and *lgl* mutants fail to pupariate and animals die after an extended larval period as giant larvae (Bilder, 2001, Bilder *et al.*, 2000, Mechler *et al.*, 1985, Woods *et al.*, 1996). Consequently, the phenotypic analysis of *scrib* and *lgl* mutant

testes was performed during the extended life of 5-7 day-old larvae. Examination of 5-7 day-old *scrib* and *lgl* mutant testes from giant larvae, revealed a dramatic reduction in their size (Fig. 7). *scrib* testes showed defects in the male stem cell niche, with less GSCs, gonialblasts and marked reduction in the transit amplifying spermatogonial cells (Fig. 7 BB'). As gonad development requires a coordinated soma-germline interaction ensuing renewal and differentiation of germline and somatic stem cells, the distribution of known nuclear markers of somatic cell differentiation, including Traffic-jam (TJ) (Li *et al.*, 2003) and Eyes absent (Eya) (Fabrizio *et al.*, 2003) was analyzed. In wt testes TJ is produced in CySCs and early SCCs wrapping 2-, 4-, 8-germ cell cysts (Fig. 7 A,A') whereas Eya is present in late SCCs enclosing larger cysts (Fig. 7D) (Hempel and Oliver, 2007). In *scrib* and *lgl* testes the number of positively stained TJ-cells was comparable to that of the wt testes (Fig. A-C). However in *scrib* testes, TJ-expressing somatic cells, marking normally the CySCs and early SCCs, were also found in association with late differentiated spermatocyte cysts containing branched fusomes (Fig. 7 B,B'). Mature spermatocyte cysts were still observed in *scrib* testes, with Eya-positive SCCs associated with late spermatogonial and spermatocyte cysts (Fig. 7E) albeit with a significantly smaller size and a more intensive Vasa staining (Fig. 7B) in comparison to the wt (Fig. 7 A,D). Although all germ cell populations, CySCs and SCCs were present in the *scrib* testis, its structure and overall size was affected with the phenotypes ranging from slightly abnormal to more severe (data not shown).

The phenotype of the *lgl* testes was more severe, with defects in the male stem cell niche, fewer GSCs loosely attached to the hub and few spermatogonial cysts with progressive spermatocyte cyst disappearance leading to testis atrophy (Fig. 7 C,C'). Spermatocyte cyst loss was also associated with an absence of Eya-positive SCCs (Fig. 7F) and only in rare cases, few surviving spermatocyte cysts containing branched fusome (an indication of cyst differentiation and maturation) were observed (Fig. 7C'). Moreover, as in the case of *dlg* testis, the number of posterior terminal cells was significantly expanded (Fig. 7 C,C'). Therefore, the phenotype of the *lgl* testis appeared to be overall very similar to the one observed previously in the *dlg* mutant testis (Papagianouli and Mechler, 2009). To summarize, Scrib and Lgl in the somatic lineage play an important role in guiding testis architecture, differentiation and development. It becomes obvious however, that *scrib* and *lgl* have slightly different functions, as *scrib* testes do not show the extensive atrophy observed in *lgl* and *dlg* testes.

Discussion

Interest towards the mechanisms leading to the formation of the germline and its differentiation during *Drosophila* development initiated already when the first ever cloned tumour suppressor gene in *Drosophila*, *lgl*, was identified (Mechler *et al.*, 1985). Yet, the dramatic overgrowth phenotypes of *lgl* as well as of the other two TSGs, Scrib (a PDZ protein of the LAP protein family) and Dlg (a PDZ protein of the MAGUK family (Woods *et al.*, 1996), captured the research focus for several

years (Bilder *et al.*, 2000, Bilder and Perrimon, 2000, Wodarz, 2000, Woods *et al.*, 1996). This work reports the role of *dlg*, *scrib* and *lgl* tumour suppressor genes in gonad formation during *Drosophila* embryogenesis and larval testis development, a period of development prior to metamorphosis, which is crucial for testis organogenesis. In particular, it is during late embryogenesis and larval development that the newly formed gonads acquire their anterior-posterior axis and initiate cell proliferation in testes, whereas ovaries remain quiescent during larval stages (Casper and Van Doren, 2006, DeFalco *et al.*, 2003). The data presented here clearly show that Dlg, Scrib and Lgl act as key regulators of gonad architecture and testis morphogenesis and are primarily required in cells of somatic origin.

Dlg and Scrib in the gonadal mesoderm affect the internal structure of the gonads

Previous analysis of Scrib distribution in embryonic gonads has revealed that zygotic Scrib (the Scrib1 larger isoform) is activated in the gonadal mesoderm at the time of gonad formation. Scrib synthesis in embryonic gonads results from the zygotic and not the maternal expression of the *scrib* gene occurring when pole cells coalesce with mesodermal cells to form the gonads (Marhold *et al.*, 2003). Scrib synthesis in the gonadal mesoderm is cell auto-

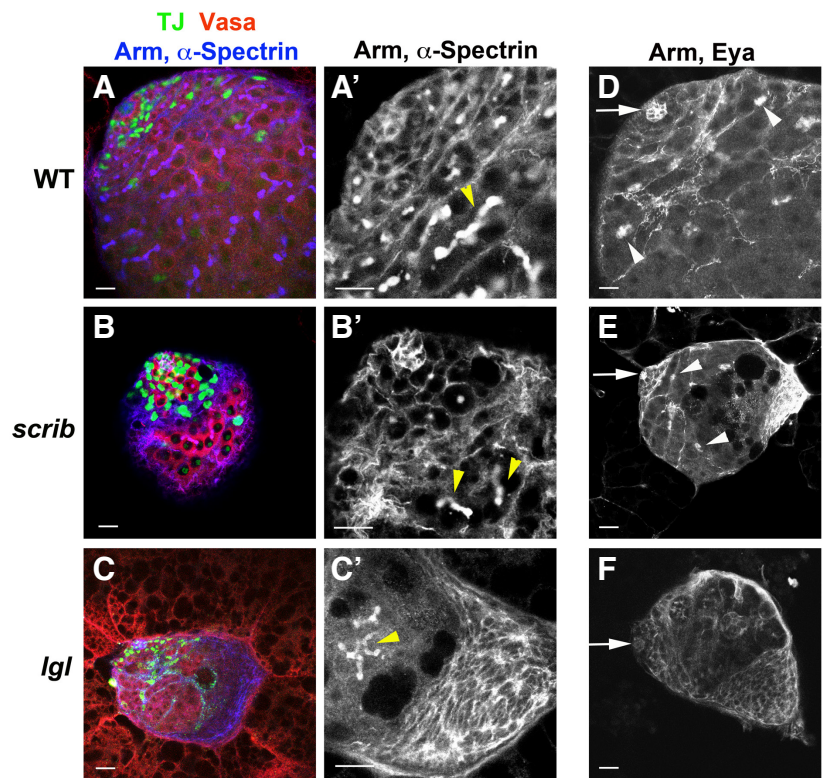


Fig. 7. Extensive defects associated with *scrib* and *lgl* mutant testes. (A-C') Testes from wt (A-A'), *scrib* (B-B') and *lgl* (C-C') mutant larvae stained for Vasa (red), TJ (green), and Arm+ α -Spectrin (blue). (A'), (B'), (C') are enlargements of the hub region from (A), (B), (C) respectively, showing only the Arm+ α -Spectrin staining. (D-F) Testes from wt (D), *scrib* (E) and *lgl* (F) larvae stained for Arm+Eya. Yellow arrows in (A', B' and C') point out the fusomes. White arrows in (D-F) point at the hub. White arrowheads in (D,E) point at Eya-positive SCCs cells, which are missing in (F). Testis hub is oriented towards the left. Scale bar, 15 μ m.

mous, as *Scrib* is present in agametic *csul^P* and *vls³* gonads made only of mesodermal cells. By contrast, *Scrib* production in pole cells requires direct contact with the gonadal mesoderm as revealed by the absence of *Scrib* in *wun* and *tin-zfh1* pseudo-gonads made only of aggregated pole cells (Marhold *et al.*, 2003).

Comparative analysis of gonadogenesis in *dlg*, *scrib* and *lgl* mutant embryos revealed that gonad formation *per se* is not affected as the gonadal mesoderm can coalesce with the germ cells, and in spite of the occurrence of misplaced pole cells the total number of pole cells per gonad remains stable. Nevertheless, *dlg* and *scrib* play a role in establishing the architecture of the newly formed gonads since they are required for the intimate contacts of the gonadal mesoderm to the pole cells. Moreover, *Scrib* requires *Dlg* for its proper localization in the embryonic gonads, which speaks for a common role and interdependence in establishing gonad architecture. In addition, *Dlg* acts critically in the incorporation of the male specific Sox100B expressing mesodermal cells in the posterior male gonads, since this group of male-specific cells is missing in newly formed *dlg* male gonads. The Sox100B transcription factor expressed in these cells is the homologue of the mammalian Sox9 protein, a critical player in human male sex determination and testis differentiation (Clarkson and Harley, 2002). Further examination of wild type and mutant larval gonads showed that Sox100B expression in the posterior mesodermal cells should be limited to the time when the testis acquires its anterior-posterior axis and initiates germ cell proliferation and differentiation (DeFalco *et al.*, 2003, Nanda *et al.*, 2009). Therefore, this study identifies *dlg*, *scrib* and their interdependence as crucial for the early development of the embryonic gonads, by exerting their function primarily through the somatic part of the gonad.

***Scrib* and *Lgl* in the somatic cyst stem cells and cyst cells are critically required for testis development and homeostasis**

The requirement of *dlg* in the gonadal mesoderm is in line with previous observations from later stages showing that *Dlg* is required in testis somatic cyst stem cells (CySCs) and early somatic cyst cells (SCCs) to establish the niche architecture, and in late SCCs for their survival and expansion, providing germ cell encapsulation and spermatogonial cyst differentiation. *dlg* testes are particularly small, with a reduced number of germ cells and degenerating spermatogonial cysts, and *dlg* expression exclusively in the somatic lineage can rescue the mutant testes (Papagiannouli and Mechler, 2009). Along this line, analysis of the phenotype in *scrib* and *lgl* depleted testes confirmed the requirement of both genes for normal male stem cell niche and testis development. *Scrib* and *Lgl* are localized in the somatic hub, CySCs and SCCs, with *Lgl* showing a stronger concentration at junctional points with neighboring germ cells. Phenotypic analysis revealed that *lgl* mutant testes look very similar to the *dlg* ones, with reduced number of GSCs loosely attached to the hub, spermatocyte cyst degeneration and very small degenerated testes, which would speak for a common function and cooperation of the two genes. The *scrib* phenotype looks quite distinct. The overall size of *scrib* testes is smaller but almost all cell types can be identified. The defects affect primarily the male stem cell niche and the transit amplifying spermatogonia, which are significantly reduced. Spermatocyte cysts with *Eya*-positive SCCs are still present but often SCC differentiation seems to be affected, as TJ-positive SCCs are associated with differentiated spermatocytes. However, *Scrib* localization in the hub cells requires *Dlg* (Papagiannouli and Mechler, 2009).

Our results from this and previous studies (Marhold, 2003) suggest that the activity of *scrib*, *dlg* and *lgl* is required after gonad formation when primarily the zygotic contribution is required. The relatively weak phenotypes observed in gonad formation, can be explained from the fact that development of the mutant defective phenotypes progresses over time, when differentiation of the gonadal mesoderm drives male gonad and subsequent testis organogenesis. Future work should aim, as in the case of *dlg*, at rescuing the mutant *scrib* and *lgl* testes phenotypes and on how testis differentiation and morphogenesis is affected at the mechanistic level. Interesting is always to see to what extent *Dlg*, *Scrib* and *Lgl* in the somatic lineage work together or act in distinct pathways in the *Drosophila* testis, as in the late embryonic gonads and the hub cells where *Scrib* depends on *Dlg* for its proper localization.

***Dlg*, *Scrib* and *Lgl* act in common pathways in various tissue contexts**

Advances in the diverse functions of *dlg*, *scrib* and *lgl* have defined them as key players in numerous tissues contents and malignancies, and revealed their multitasking role in: junction and cytoskeleton establishment, epithelial cell and planar cell polarity; asymmetric neuroblast division and neuromuscular junctions; cancer initiation and metastasis; cooperation with various signaling pathways in different tissue contexts (Lecuit and Wieschaus, 2002, Papagiannouli and Mechler, 2012). Similar to other tissues *dlg*, *scrib* and *lgl* play an important role in testis development in the various populations and their cooperation according to the developmental stage can vary significantly. This study, together with the recent advances on the function of these genes uncovers the diverse, multitasking role of these genes as key players in the cell-type and tissue specific contexts. Analysis of the degree of their interdependence in the *Drosophila* testis at the mechanistic level will provide new insights on the role of these genes in testis architecture and in maintaining the integrity and microenvironment of the cysts.

The results on the role of *scrib* and *lgl* shown here and of previous results on *dlg* in the *Drosophila* testis (Papagiannouli and Mechler, 2009, Papagiannouli and Mechler, 2010) are in agreement with the newly investigated role of these genes in the cancer field. Nowadays, the cellular context and the neighbouring cells of the surrounding tumour environment are recognized as important regulators of cancer progression (Brumby and Richardson, 2005, Humbert *et al.*, 2008, Mohamet *et al.*, 2011, Pagliarini and Xu, 2003, Schmeichel, 2004, Woodhouse and Liotta, 2004). The results obtained in the cancer field and testis, concerning the role of the microenvironment created between juxtaposed cells types point out possible similarities of the basic mechanisms underlying the function of these genes. The effect of *Dlg*, *Scrib* and *Lgl* when signaling pathways emanating from the somatic and germ cells are affected, and the comparative analysis of apoptosis induction in the testis and in mosaic clones of the tumor microenvironment are interesting aspects for further investigation. Answering these questions will help us understand how the cell-type specific cellular content (cell intrinsic effects), the microenvironment and signaling pathways cooperate with *dlg*, *scrib* and *lgl* in the various tissues.

Materials and Methods

Oregon R was used as a wild type stock. The following mutant stocks have been used in this study: *dlg^{m52}*, *lgl⁴* and *scrib^{P^{var}}* (De Lorenzo *et al.*, 1999, Li

et al., 2001, Marhold *et al.*, 2003, Papagiannouli and Mechler, 2009). Immunostainings in *Drosophila* embryos and testes, confocal imaging and picture processing are previously described (Papagiannouli and Mechler, 2009). The following antibodies were used: mouse monoclonal anti-Dlg (1/100; gift from P. Bryant), polyclonal rabbit anti-Scrib (1/200; (Li *et al.*, 2001)) and polyclonal rabbit anti-Lgl (1/200; (Strand *et al.*, 1994a)). The mouse monoclonal anti-Armadillo N7A1 (1/10), anti-FasIII (1/100), anti-eya10H6 (1/100), anti- α -spectrin (3A9) (1/100), anti-Neurotactin-BP106 (1/100) and anti-Vasa (1/10) 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. 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). The guinea pig polyclonal anti-Traffic-jam (TJ) antibodies (1:2000) and the rabbit polyclonal anti-Sox100B antibodies (1:1000) were a gift of D. Godt and S. Russell, respectively. The *Scrib*^{CA07683}-GFP and *Lgl*^{BA00193}-GFP trap lines were obtained from the "FlyTrap-GFP Protein Trap Database" (Buszczak *et al.*, 2007, Kelso *et al.*, 2004).

Homozygous mutant embryos were distinguished from heterozygous embryos carrying a *ftz-lacZ* gene on the FM7 balancer chromosome in the case of *dlg*, *actin-lacZ* on the SM6 balancer chromosome for *lgl* and *ftz-lacZ* on the TM3 balancer chromosome for *scrib*^{P^{varul}}. As the *dlg* gene is on the X chromosome, all mutant *dlg* embryos and testes from giant larvae of the genotype *dlg*^{m52}/FM7 are male. *dlg*, *scrib* and *lgl* mutant 3rd instar larvae are giant, bloated with melanotic spots and can be very easily identified.

Acknowledgements

I would like to thank the *Drosophila* community for providing generously fly stocks and antibodies, in particular D. Godt for the guinea pig anti-TJ and S. Russell for the rabbit anti-Sox100B antibodies, Chris Doe for the *dlg*^{m52} fly stock, Bernard M. Mechler for the *lgl*⁴ and the *scrib*^{P^{varul}} fly stocks and Joachim Marhold for confocal assistance. I am grateful to Bernard M. Mechler for his constant support throughout these years. My apologies to those people, whose work has not been cited due to space limitations.

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5 yr ISI Impact Factor (2011) = 2.959

