The receptor tyrosine phosphatase Lar regulates adhesion between \textit{Drosophila} male germline stem cells and the niche

Shrividhya Srinivasan\textsuperscript{1}, Anthony P. Mahowald\textsuperscript{2} and Margaret T. Fuller\textsuperscript{1,*}

SUMMARY

The stem cell niche provides a supportive microenvironment to maintain adult stem cells in their undifferentiated state. Adhesion between adult stem cells and niche cells or the local basement membrane ensures retention of stem cells in the niche environment. \textit{Drosophila} male germline stem cells (GSCs) attach to somatic hub cells, a component of their niche, through E-cadherin-mediated adherens junctions, and orient their centrosomes toward these localized junctional complexes to carry out asymmetric divisions. Here we show that the transmembrane receptor tyrosine phosphatase Leukocyte-antigen-related-like (Lar), which is best known for its function in axonal migration and synapse morphogenesis in the nervous system, helps maintain GSCs at the hub by promoting E-cadherin-based adhesion between hub cells and GSCs. Lar is expressed in GSCs and early spermatogonial cells and localizes to the hub-GSC interface. Loss of Lar function resulted in a reduced number of GSCs at the hub. Lar function was required cell-autonomously in germ cells for proper localization of Adenomatous polyposis coli 2 and E-cadherin at the hub-GSC interface and for the proper orientation of centrosomes in GSCs. Ultrastructural analysis revealed that in Lar mutants the adherens junctions between hub cells and GSCs lack the characteristic dense staining seen in wild-type controls. Thus, the Lar receptor tyrosine phosphatase appears to polarize and retain GSCs through maintenance of localized E-cadherin-based adherens junctions.

KEY WORDS: Adult stem cells, \textit{Drosophila} male germline, Receptor tyrosine phosphatase Lar, Stem cell-niche adhesion

INTRODUCTION

Many adult stem cells that maintain and repair tissues throughout the life of an organism reside in a specialized microenvironment, termed the stem cell niche, that helps retain stem cells in an undifferentiated state via short-range signaling (Jones and Wagers, 2008; Morrison and Spradling, 2008). The ability of adult stem cells to recognize and establish adhesion to the niche is crucial for long-term maintenance of adult stem cells. The ability to recapitulate and harness these mechanisms will be important for the use of adult stem cells in regenerative medicine. Attachment of stem cells to the niche also plays a role in orienting the axis of stem cell divisions, allowing for either asymmetric divisions to give rise to stem cells and differentiating daughter cells, or symmetric divisions to expand the stem cell pool (Knoblich, 2008; Lin, 2008; Morrison and Kimble, 2006). Cell adhesion molecules such as cadherins and integrins have been identified as being upregulated or important in adult stem cells in several systems (Ellis and Tanentzapf, 2010; Raymond et al., 2009), underscoring the importance of stem cell-niche or stem cell-extracellular matrix attachments in maintaining niche structure, retaining stem cells in the niche, and orienting stem cell divisions (Marthiens et al., 2010).

E-cadherin-based stem cell-niche adhesion has been shown to play an important role in the maintenance of \textit{Drosophila} germline stem cells (GSCs) in their niche (Song et al., 2002; Voog et al., 2008; Wang et al., 2006). Two populations of stem cells reside at the apical tip of \textit{Drosophila} testes: GSCs, which differentiate to give rise to sperm; and somatic cyst stem cells (CySCs), which constitute an important component of the GSC niche (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010) and give rise to the cyst cells that envelop and ensure the proper differentiation of germ cells (Kiger et al., 2000; Sarkar et al., 2007; Tran et al., 2000). GSCs and CySCs are associated with, and organized around, a tight cluster of postmitotic somatic cells called the hub (Hardy et al., 1979). The hub contributes to the niche by secreting the cytokine Unpaired (Upd; Outstretched – FlyBase), which locally activates the Janus kinase-Signal transducer and activator of transcription (JAK-STAT) pathway in the stem cells, maintaining GSC attachment to the hub and preventing CySC differentiation (Kiger et al., 2001; Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001).

GSCs attach to the hub cells through adherens junctions, and components of the adherens junctions, which include E-cadherin (Shotgun – FlyBase) and Armadillo (Arm; fly β-catenin), are concentrated at the interface between GSCs and hub cells as well as between adjacent hub cells (Yamashita et al., 2003). Marked GSCs that lack E-cadherin function, induced by mitotic recombination, fail to be maintained at the hub (Voog et al., 2008). E-cadherin in GSCs might contribute to stem cell maintenance by promoting GSC adhesion to the hub, so that GSCs continue to receive Upd signals and are flanked by CySCs. The adherens junctions between GSCs and hub cells also polarize GSCs by recruiting one of the fly homologs of mammalian adenomatous polyposis coli (APC), Apc2, to the cortex adjacent to the hub-GSC interface in GSCs (Yamashita et al., 2003). The localized adherens junctions and Apc2, in turn, maintain the stereotypic orientation of the mother centrosome toward the hub, which sets up the strictly oriented mitotic spindle and enables the normally asymmetric outcome of GSC divisions (Inaba et al., 2010; Yamashita et al., 2003; Yamashita et al., 2007). When a GSC divides, one of the daughter cells inherits the adhesion with the hub and retains stem...
cell fate. The daughter displaced from the hub becomes a gonialblast and initiates the differentiation program, embarking on four rounds of transit-amplification divisions with incomplete cytokinesis, then switching to spermatocyte fate, meiosis and spermatid differentiation (Davies and Fuller, 2008). The gonialblast and the transit-amplifying spermatogonial cells have the capacity to dedifferentiate to GSC fate and reoccupy the niche (Brawley and Matunis, 2004; Cheng et al., 2008; Sheng et al., 2009), although the mechanism by which dedifferentiating germ cells recognize and form attachment to the niche is not known.

Here we discover a new player in the mechanism that maintains GSCs in their niche in Drosophila testes: the receptor tyrosine phosphatase Leukocyte-antigen-related-like (Lar), which is best known for its role in axonal migration, target selection and synapse formation in the nervous system (Baker and Macagno, 2010; Chagnon et al., 2004; Johnson and Van Vactor, 2003; Sethi et al., 2010). Drosophila Lar is a transmembrane type IIa receptor protein tyrosine phosphatase that contains N-terminal immunoglobulin-like domains and membrane-proximal fibronectin type III repeats in its extracellular region, a single-pass transmembrane domain and two tandem repeats of protein tyrosine phosphatase domains in its cytoplasmic region (Chagnon et al., 2004). Lar expression is upregulated in a microarray analysis of testes with elevated numbers of early germ and cyst cells (D. L. Jones, E. L. Davies and M.T.F., unpublished results). Our data suggest that Lar, expressed in GSCs and early germ cells, maintains GSCs at the hub by promoting E-cadherin-based adhesion between hub cells and GSCs.

MATERIALS AND METHODS

Fly husbandry and stocks

All stocks were grown on standard cornmeal/molasses/yeast/agar medium at 25°C unless otherwise stated. Strains are described in FlyBase (http://flybase.org) or otherwise specified. y′ w′ flies were used as wild-type controls, except that Oregon R flies were used as wild-type controls for the ultrastructural analysis. Flies used in this study include the following stocks carrying Lar alleles: (1) w; Lar431, FRT40A/CyoO, P[Ubi-GFP.S65T], an allele with an uncharacterized molecular lesion [gift from T. Clandinin (Clandinin et al., 2001)]; (2) w; Lar2177, FRT40A/CyoO, P[Ubi-GFP.S65T], a loss-of-function allele with a nonsense mutation that truncates Lar protein in the sixth fibronectin domain (Maurel-Zaffran et al., 2001); and (3) Df(2L)E55, rdo1, hk1, Lar235, prs/CyoO, P[Ubi-GFP.S65T], a deficiency that deletes the 5’ portion of the lar gene [Bloomington Drosophila Stock Center (BDSC)]; and the following stocks carrying era alleles: (1) w; FRTG13, era210/CyoO and (2) w; FRTG13, era215/CyoO; both alleles harbor point mutations in domains crucial for Era function (Ahern-Djami et al., 1998). The stocks (1) y″ w″, hs-FLP22; FRT40A, Ubi-GFP/CyoO, (2) y″ w″, hs-FLP22; FRT40A, arm-lacZ/CyoO and (3) y″ w″, hs-FLP22; FRTG13, Ubi-GFP/CyoO (BDS) were paired with the above stocks for inducing mutant clones or paired with FRT40A/CyoO or FRTG13/CyoO (BDS) for inducing wild-type control clones. 

Immunofluorescence

Testes were dissected in PBS and fixed for 20 minutes in 4% formaldehyde in PBS. To stain with antibodies, testes were permeabilized for 1 hour in PBS with 0.3% Triton X-100 and 0.6% sodium deoxycholate at room temperature, washed in PBST (0.1% Triton X-100 in PBS), blocked in PBST with 3% BSA for 1 hour at room temperature or overnight at 4°C and either incubated overnight at 4°C or for 2 hours at room temperature in primary antibody diluted in PBST with 3% BSA. The testes were washed in PBST, incubated in appropriate fluorochrome-conjugated secondary antibodies raised in donkey (1:500; Jackson Labs) for 2 hours at room temperature, again washed with PBST, and mounted in Vectashield containing DAPI (Vector Laboratories).

Antibodies used in this study include those obtained from Drosophila Studies Hybridoma Bank against the extracellular N-terminal domain of Lar [mouse, 1:10 (Sun et al., 2000)], Arm [mouse, 1:10 (Riggelman et al., 1990)], Spectrin [mouse, 1:10 (Dubreuil et al., 1987)], Enabled [mouse, 1:10 (Bashaw et al., 2000)], Fas III [mouse, 1:10 (Patel et al., 1987)] and E-cadherin [rat, 1:10 (Oda et al., 1994)]. Other antibodies used include those against γ-Tubulin (mouse, 1:50; Sigma), β-galactosidase (mouse, 1:100; Sigma), Traffic jam [rat and guinea pig, 1:500; a gift from D. Godt (Li et al., 2003)], Vasa (goat, 1:50-1:100; DC-13 from Santa Cruz Biotech), green fluorescent protein (GFP) (rabbit, 1:500-1:1000, Invitrogen; sheep, 1:1000, AbD Serotec), Apc2 (rabbit, 1:5000; a gift from M. Bienz (Yu et al., 1995)), Lar (rabbit, 1:500-5000; a gift from R. Lehmann) and Stat92E (rabbit, 1:1000; a gift from E. Bach, NYU School of Medicine, NY, USA) (Flaherty et al., 2010).

Images were captured using a Leica SP2 AOBs confocal laser-scanning microscope and processed using Adobe Photoshop CS5 software. GSCs were scored either as Vasa-positive cells adjacent to the hub (detected using Arm or E-cadherin) and containing dot spectromers (detected using Spectrin) or as cells that are negative for Traffic jam staining and adjacent to the hub.

Clonal analysis

Homozygous mutant clones in a heterozygous background were induced using Flippase (FLP)-mediated mitotic recombination, y,w hs-FLP22; FRT40A/CyoO, FRTG13, Ubi-GFP virgin females were crossed to w; FRT40A or w; Lar431, FRT40A/CyoO or w; Lar2177, FRT40A/CyoO males. Similarly, y,w hs-FLP22; FRTG13, Ubi-GFP virgin females were crossed to w; FRTG13 or w; era210; FRTG13/CyoO or w; era215, FRTG13/CyoO males to generate homozygous era mutant clones. To compare E-cadherin-GFP localization in GSCs that were heterozygous and homozygous mutant for Lar function, hs-FLP22; FRT40A, arm-lacZ/CyoO; NGVP16 virgins were crossed to either w; Lar431, FRT40A/CyoO; UAS-DEFL-#6-1 or w; Lar2177, FRT40A/CyoO; UAS-DEFL-#6-1 males. The progeny were grown until the pupal stage at 25°C, then heat shocked at 37°C for 2 hours on two consecutive days. Testes were dissected from flies of appropriate genotypes on different days after the second day of heat shock and examined for the presence of GFP-negative clones in germ cells by fluorescence microscopy. Lar and era mutant GSC clones were identified by immunostaining with antibodies against GFP, Vasa and Arm. CySC clones were identified with antibodies against GFP and Traffic jam. E-cadherin-GFP expression in Lar mutant GSC clones was detected using antibodies against GFP and counterstained with anti-β-galactosidase to identify marked clones and with anti-E-cadherin and anti-Traffic jam to identify GSCs. All clonal analyses were repeated independently at least three times and the data are shown as an average of the independent experiments. Analysis of Stat92E accumulation, Apc2 localization, centrosome orientation and E-cadherin-GFP localization were performed in testes 5 days post-clone induction, which is 3 days after eclosion of adult males.

Ultrastructural analysis of adherens junctions in GSCs

Testes were dissected and incubated in fixative (2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2) at room temperature for 15 minutes, then transferred to ice in this solution for up to 1 hour. Testes were postfixed with 2% OsO4 for 2 hours at 4°C, then transferred to 1% uranyl acetate overnight at 4°C. Tissues were dehydrated in ethanol and embedded in Epon resin. Serial sections (80 nm) were picked up on formvar-covered wire loops, transferred to slot grids and stained with uranyl acetate and lead citrate. Sections were viewed with a Tecnai F30 electron microscope at 300 kV and images recorded on a Gatan digital camera. The membrane interface for each hub-GSC combination was scanned over multiple sections to identify the presence of adherens junctions.
RESULTS
Lar is expressed in early germ cells and localizes to the hub-GSC interface
Immunofluorescence analysis using antibodies against Lar revealed expression of Lar protein at the apical tip of adult (Fig. 1A,A') and third instar larval (data not shown) wild-type testes. Examination of testes in which early germ cells were marked by the expression of GFP-tagged α-Tubulin revealed anti-Lar immunofluorescence in GSCs, where it localized to the membrane at the interface between the hub and GSCs (arrowheads, Fig. 1A,A',B'). Lar was also detected in two- and four-cell spermatogonial cysts, with only a weak signal in eight-cell spermatogonial cysts, where it localized to the membrane between mitotic sister germ cells of a cyst (arrows, Fig. 1A,A'). Lar signal was below detection in CySCs (arrowheads, Fig. 1C'), somatic cyst cells, and in germ cells in later stages of differentiation.

Lar helps maintain GSCs at the hub
Adult escapers of a heteroallelic combination of Lar alleles, Lar451/Lar2127, survived for 2-3 days post-eclosion. Examination of testes isolated from newly eclosed Lar451/Lar2127 males by phase-contrast microscopy revealed a normal progression of germ cell differentiation, similar to that seen in wild-type testes (data not shown), suggesting that Lar function is not necessary for germ cell differentiation. Although reproductive tracts from Lar mutant males had motile sperm, Lar451/Lar2127 males were sterile. Lar staining was not detected at the apical tip in testes isolated from 0- to 1-day-old Lar451/Lar2127 adult males (Fig. 2B,B') or from larval testes (data not shown), suggesting that the antibodies used specifically detected Lar protein in wild-type testes (Fig. 2A,A') and that the allelic combination is a protein null.

Examination of Lar mutant adult or larval testes immunostained with antibodies against Vasa to mark germ cells, Zfh1 to mark early cyst cell nuclei and Arm to mark the hub, revealed a defect in GSC maintenance. The mutant testes had fewer GSCs adjacent to the hub compared with wild type (Fig. 2C,D; data not shown for larval testes). In addition, although in wild-type testes Zfh1-positive nuclei of cyst cells were found one GSC diameter away from the hub (arrows, Fig. 2C') (Leatherman and Dinardo, 2008), in Lar mutant testes Zfh1-positive cyst cells were positioned adjacent to the hub in regions where GSCs were lacking (arrows, Fig. 2D').

Quantitative assessment revealed that wild-type adult testes had an average of 8.06±1.58 GSCs per testis, whereas testes isolated from Lar451/Lar2127 mutant adults averaged 3.35±1.52 GSCs per testis (Fig. 2E). A similar reduction in GSC numbers was also observed in Lar451/Lar2127 mutant larval testes (5±1.5 GSCs per testis in Lar mutants compared with 9.1±1.2 GSCs per testis in wild type; data not shown), suggesting that fewer GSCs are maintained adjacent to the hub. Testes isolated from Lar2127/Df(2L)E55 adult escapers also averaged 3.14±1.29 GSCs per testis (Fig. 2E). Interestingly, adult testes from Lar mutant alleles heterozygous with wild type (Lar451/+ or Lar2127/+)) also showed a slight, but statistically significant, reduction in GSC numbers compared with wild-type controls (Fig. 2E), suggesting that Lar function to maintain GSCs might be dose dependent. The number of Zfh1-positive cyst cell nuclei within one cell diameter of the hub was slightly reduced in testes isolated from Lar451/Lar2127 mutant adults (13±3.1 CySCs per testis; n=52 testes) compared with wild type (16.95±3.4 CySCs per testis; n=60 testes) (P<0.0001, two-tailed Student’s t-test). Since Lar protein expression was not detected in CySCs (Fig. 1C), the slight reduction in the number of Zfh1-positive cells in Lar mutants might be due to indirect effects on the cyst cells.

Lar functions cell-autonomously to maintain GSCs
Clonal analysis revealed that Lar function is required cell-autonomously for GSC maintenance, but not for germline differentiation. Negatively marked homozygous Lar mutant clones were generated for two different Lar alleles using the FLP-FRT-based mitotic recombination system in a Lar heterozygous background. Lar protein was not detected in GSCs homozygous for either the Lar451 or the Lar2127 allele by immunostaining 5 days post-clone induction (PCI) (supplementary material Fig. S1A,B), suggesting that both alleles of Lar are protein null. By day 3 PCI, only 46.5% of testes carrying the Lar451 allele and 38% of testes carrying the Lar2127 allele had at least one marked homozygous mutant GSC compared with 85.6% testes in the wild-type control (Fig. 2F). Under the same conditions of clone induction, at day 3 PCI germ cells in later stages of differentiation that were homozygous mutant for either of the two Lar alleles (GFP negative) were present at levels similar to the wild-type controls (Fig. 2G), suggesting that the low percentage of testes with Lar mutant GSCs was not due to a defect in clone induction. GFP-negative spermatocytes and round or elongated spermatids were present 7 days PCI (supplementary material Fig. S1C-H), suggesting that, at least until the spermatid stage, Lar function might not be required for germ cell differentiation. The percentage of testes having marked Lar451 or Lar2127 mutant GSCs steadily decreased to ~20% by day 15 PCI, suggesting that Lar functions cell-autonomously to maintain GSCs.
Fig. 2. Lar functions cell-autonomously to maintain GSCs at the hub. (A-B’/A-D’) Apical tips of 0- to 1-day-old adult Drosophila testes stained with antibodies against Lar (green in A, B, white in A’, B’), co-stained for E-cadherin (red) to mark the hub and with DAPI (magenta) to mark DNA in (A) wild type and (B) Lar451/Lar2127 mutant. GSCs are outlined. Arrows indicate that Lar is expressed and localizes to the hub-GSC interface in wild type (A’), but not in the mutant (B’). (C-D’/C’-D’) Wild-type (C, C’) and Lar451/Lar2127 mutant (D, D’/A-D’/A-D’) adult testes immunostained with anti-Vasa (red) to label germ cells, anti-Arm (magenta) to mark the hub and (C’, D’, arrows) anti-Zfh1 (green) to label early cyst cell nuclei. (E) Quantitation of the number of GSCs adjacent to the hub per testis, reported as average ± s.d. N, the number of testes scored for each genotype. P-values of Student’s t-test (two-tailed) are shown. (F-H) Germine clonal analysis of the Lar mutant alleles Lar451 (red) and Lar2127 (green) compared with FRT40A controls (blue). The reduction in the number of GFP-negative Lar mutant GSCs maintained (F), the decrease in Lar mutant spermatocyte cysts and spermatids (G) and the maintenance of CySCs (H) scored at different time points post-clone induction (PCl). Numbers next to the data points indicate the number of testes scored for each genotype at the different time points. The same numbers of testes were scored for F and H. Data are average ± s.d. Scale bars: 25 μm.

By day 15 PCl, similar to the reduction in the percentage of testes with marked Lar mutant GSC clones, the percentage of testes with negatively marked clones of spermatocytes and later stages of germ cell differentiation also dropped to ~20%, likely reflecting the corresponding loss of marked GSCs (Fig. 2G). Consistent with the inability to detect Lar protein by immunostaining in CySCs and cyst cells, Lar mutant CySC clones were maintained at similar levels as wild-type control clones (Fig. 2H), indicating that Lar function is not required cell-autonomously for CySC maintenance.

A known substrate of the phosphatase activity of Lar, Enabled (Ena), localizes to the hub-GSC interface. However, Ena is not absolutely required for GSC maintenance. Drosophila Ena, a member of the Ena/VASP family of filamentous actin (F-actin) regulators, physically and genetically interacts with Lar and serves as substrate for Lar in vitro (Wills et al., 1999). Immunostaining with anti-Ena in wild-type testes revealed localization of Ena to the hub-GSC interface (arrows, Fig. 3A’), similar to Lar localization. Examination of Ena localization in Lar451/Lar2127 mutant GSCs revealed that Ena failed to localize at the hub-GSC interface in about half the GSCs (arrowhead, Fig. 3B’). Of the 145 Lar451/Lar2127 mutant GSCs scored, 51.7% had Ena distribution throughout the GSC cortex, as compared with 18.7% in wild-type GSCs (171 wild-type GSCs were scored).

To test the possibility that Lar might act through Ena in GSCs, with failure of Ena localization at the hub-GSC interface a possible cause of GSC loss, the maintenance of GSCs upon loss of ena function was determined. GSCs homozygous mutant for the ena loss-of-function alleles ena23 and ena210 were generated using the FLP-FRT system and scored for maintenance at different time points PCI. At 2 days PCl, 83.5% of testes carrying the ena23 allele and 90% of testes carrying the ena210 allele had at least one marked homozygous mutant GSC, comparable to wild-type controls (100% testes) (Fig. 3C). The percentage of testes with ena210 homozygous mutant GSC clones dropped from 90% at day 2 PCl to 56% at day 3 and to 45.5% by day 15 PCl, as compared with 100% (day 3) to 95.2% (day 15) in wild-type controls. The percentage of testes containing marked GSC clones homozygous mutant for ena210 did not drop significantly over time, remaining at 73.5% at day 15 PCl. Thus, unlike marked Lar mutant GSC clones, which were steadily lost over time (Fig. 2F), ena mutant GSCs, after an initial loss for one of the alleles examined, were maintained, suggesting that ena might not be the sole or key downstream target of Lar for the maintenance of GSCs at the hub.

Lar function is not required for Stat92E accumulation in GSCs

Immunofluorescence staining for expression of Stat92E, an indicator of JAK-STAT signaling activity (Chen et al., 2002), revealed that Lar was not required for the accumulation of Stat92E protein in the GSCs that remained next to the hub. In wild-type testes, Stat92E is expressed in GSCs, CySCs and, to a lesser extent, in some gonialblasts (Fig. 4A, A’). The GSCs remaining at the hub in Lar451/Lar2127 adult testes had Stat92E protein in the cytoplasm and the nucleus (Fig. 4B, B’). Stat92E protein was also detected in
Vasa-positive cells displaced away from the hub in Lar^{451}/Lar^{2127} testes (long arrow, Fig. 4B), suggesting that the loss of GSCs was not due to loss of JAK-STAT signaling in Lar mutants. Similarly, Stat92E levels in Lar^{451} or Lar^{2127} homozygous mutant GSCs were similar to those in the neighboring heterozygous GSCs (Fig. 4C,D). Relative levels of Stat92E protein in CySCs versus GSCs were similar in Lar mutants and wild-type controls (arrowheads, Fig. 4A,B). Loss of GSCs in Lar mutants was unlikely to be due to changes in JAK-STAT-mediated competition by CySCs as previously described (Issigonis et al., 2009), where loss of Suppressor of cytokine signaling at 36E function, a negative regulator of the JAK-STAT pathway, resulted in upregulation of Stat92E in CySCs relative to neighboring GSCs, causing CySCs to outcompete GSCs for attachment to the hub.

**Localization of Apc2 to the hub-GSC interface and proper orientation of centrosomes in GSCs require Lar function**

The GSCs remaining at the hub in Lar mutant testes tended to exhibit abnormal localization of Apc2 protein. *Drosophila* Apc2 is enriched at the cortical region of GSCs adjacent to the hub-GSC interface (Fig. 5A,A') (Yamashita et al., 2003). Immunostaining with anti-Apc2 revealed that, in 57.5% of the GSCs that remained adjacent to the hub in 0- to 1-day-old Lar^{451}/Lar^{2127} testes, the Apc2 detected was distributed around the GSC cortex, rather than localized to the hub-GSC interface (Fig. 5B,B'). By contrast, only 21.2% of GSCs in wild-type testes had anti-Apc2 signal distributed around the GSC cortex (Fig. 5E). Furthermore, 76.6% of homzygous Lar^{451} and 57% of homzygous Lar^{2127} mutant GSCs at 5 days PCI also exhibited Apc2 distributed over the GSC cortex (Fig. 5C,D,F) compared with 27.3% and 23.8% of the neighboring heterozygous control GSCs (arrowheads). Homozygous mutant GSCs (green outline in C',D') are detected by the absence of anti-GFP immunostaining (green, C,D; white C',D'). The star indicates the Hub. Scale bars: 25 μm.

**Fig. 3. Ena function is not absolutely required for GSC maintenance.** (A-B') 0- to 1-day-old wild-type (A,A') and Lar^{451}/Lar^{2127} mutant (B,B') adult *Drosophila* testes immunostained with anti-Ena (green, A,B; white A',B'), co-stained with anti-Vasa (red) to mark germ cells and anti-E-cadherin (magenta) to mark the hub. Arrows (A',B') indicate Ena localization to the hub-GSC interface. Arrowhead (B') indicates an example where Ena failed to localize at the hub-GSC interface. Ena failed to localize to the hub-GSC interface in 37.8% of the total GSCs with two centrosomes, compared with 11.9% of GSCs in wild-type controls (Fig. 5G). Similarly, in homozygous Lar^{451} and Lar^{2127} mutant GSCs remaining at the hub in 0- to 1-day-old Lar^{451}/Lar^{2127} mutant testes (Fig. 5H) showed an increased percentage of misoriented centrosomes compared with their controls. In 0- to 1-day-old Lar^{451}/Lar^{2127} adults, neither of the centrosomes was adjacent to the hub-GSC interface in 37.8% of the total GSCs with two centrosomes, compared with 11.9% of GSCs in wild-type controls (Fig. 5G). Similarly, in homozygous Lar^{451} and Lar^{2127} mutant...
GSCs at 5 days PCI, 52% and 48.8% of homozygous mutant GSCs exhibited misoriented centrosomes, respectively, as compared with 14.4% and 12% in neighboring heterozygous GSCs (Fig. 5H).

**Defective adherens junctions between hub cells and GSCs in Lar mutants**

The hub-GSC interface, where Lar localizes, is enriched in adherens junctions (Yamashita et al., 2003), raising the possibility that Lar affects hub-GSC adhesion. Ultrastructural analysis revealed abnormal adherens junctions between GSCs and hub cells in Lar mutant testes. Adherens junctions between GSCs and the hub, as analyzed for eight GSCs from three wild-type testes by transmission electron microscopy, appeared as electron-dense structures between the opposing cell membranes (Fig. 6A). By contrast, of the nine Lar mutant GSCs from five testes analyzed, none had strong electron-dense adherens junctions between hub and GSCs (Fig. 6B). Instead, in regions where the plasma membranes between hub cell and an adjacent GSC were closely opposed, the intervening material displayed only weakly electron-dense staining (compare magnified images 1, 2 and 3 in Fig. 6A with 4, 5 and 6 in 6B), suggesting that adherens junctions between hub cell and GSCs in Lar mutant testes might be weak compared with those in wild-type controls.

Interestingly, the membranes between adjacent hub cells also exhibited weakly electron-dense staining in Lar mutant testes as compared with wild-type controls (data not shown). The localization of E-cadherin (supplementary material Fig. S3B) and Arm (Fig. 2D') between hub cells of Lar<sup>551</sup>/Lar<sup>2127</sup> mutant testes and Arm<sup>H11032</sup> mutant GSCs from five testes analyzed, were reduced in Lar<sup>551</sup>/Lar<sup>2127</sup> mutant GSCs (arrowhead, Fig. 6D<sub>C</sub>-<sub>D</sub>) suggesting a possible role for Lar in the hub in addition to its germ cell-autonomous roles.

**Retention of GFP-tagged E-cadherin at the hub-GSC interface is affected in Lar mutant GSCs**

The reduction in the number of GSCs retained at the hub, the mislocalization of Apc2 around the GSC cortex and the abnormal appearance of adherens junctions by ultrastructural analysis in Lar loss-of-function mutants suggest that Lar might help maintain hub-GSC adhesion, possibly through effects on E-cadherin, a major structural component of adherens junctions (Harris and Tepass, 2010) that is present at the membranes between hub and GSCs in wild-type testes (arrow, Fig. 6C<sub>D</sub> - H<sub>D</sub>). Although anti-E-cadherin staining revealed localization at the hub-GSC interface in some GSCs in 0- to 1-day-old Lar<sup>551</sup>/Lar<sup>2127</sup> mutant testes, similar to in wild type (arrow, Fig. 6D'), levels of E-cadherin staining were lower or missing altogether in other Lar mutant GSCs (arrowhead, Fig. 6D').

As E-cadherin present on GSC membranes is difficult to distinguish from that present on the adjacent hub cell membranes when the two are juxtaposed at the hub-GSC interface, the...
localization of E-cadherin in GSCs was examined by expressing UAS-E-cadherin-GFP specifically in the germ cells under the control of the NGVP16 driver. E-cadherin-GFP expression by the UAS-GAL4 system in Lar mutant GSCs failed to rescue the loss of GSCs in Lar\(^{451}/Lar^{2127}\) heterozygous mutant testes. In addition, E-cadherin failed to localize at the membrane in Lar mutant GSCs. When E-cadherin-GFP was expressed in wild-type (Fig. 7A,A') or Lar heterozygous (Fig. 7B,B') GSCs using the NGVP16 driver, the GFP-tagged protein localized to the hub-GSC interface, consistent with the localization of adherens junctions between the hub and GSCs (Inaba et al., 2010; Yamashita et al., 2003). By contrast, E-cadherin-GFP did not localize to the hub-GSC interface in the GSCs that remained next to the hub in Lar mutant testes. Of the 87 GSCs analyzed from 27 Lar mutant testes, only six GSCs exhibited cortical expression of E-cadherin-GFP. Of these, three GSCs exhibited E-cadherin-GFP localization to the hub-GSC interface. Strikingly, in the remaining 81 Lar mutant GSCs no E-cadherin-GFP expression was detected (Fig. 7C,C').

The ability of some GSCs to persist next to the hub in Lar mutant testes might be due to partial redundancy between Lar and other tyrosine phosphatases such as the type IIA family receptor tyrosine phosphatase Ptp69D, which has overlapping functions with Drosophila Lar in the central nervous system and the visual cortex and shares common signaling mechanisms (Clandinin et al., 2001; Desai et al., 1997; Garrity et al., 1999; Krueger et al., 1996; Maurel-Zaffran et al., 2001). Alternatively, weak hub-GSC adhesion in Lar mutant testes might enable CySCs to compete for attachment to the hub, displacing some, but not all, GSCs from the hub. CySCs normally have smaller regions of contact with the hub than do GSCs (Hardy et al., 1979) but can outcompete GSCs from the hub when provided with an advantage. For example,
overexpression of components of the integrin-based adhesion system in CySCs resulted in displacement of GSCs from the hub by CySCs (Issigonis et al., 2009).

In wild-type testes, Lar localizes to the hub-GSC interface, which is the region of cell cortex where localized adherens junctions anchor GSCs to their niche (Yamashita et al., 2003). Adherens junctions are formed by extended clustering of transmembrane cadherin proteins that form homotypic interactions with cadherins on opposing cell membranes (Harris and Tepass, 2010). The highly conserved cytoplasmic tail of E-cadherin acts as an anchor for β-catenin and p120-catenin and indirectly for α-catenin through its interaction with β-catenin (Pokutta and Weis, 2007). Lar also localizes to adherens junctions in epithelial cells (Aicher et al., 1997) and in neuronal synapses that are enriched in cadherin-catenin complexes (Dunah et al., 2005). Lar physically associates with the cadherin-catenin complex in cultured cells (Aicher et al., 1997; Kypta et al., 1996; Muller et al., 1999) and with N-cadherin in Drosophila embryos (Prakash et al., 2009).

Adherens junctions are associated with underlying arrays of cortical F-actin (Hirokawa et al., 1983), organized by the high local concentration of α-catenin dimers (Drees et al., 2005; Weis and Nelson, 2006). F-actin filaments, in turn, regulate the stability and strength of adherens junctions (Chu et al., 2004; Ehrlich et al., 2002; Hansen et al., 2002; Jamora and Fuchs, 2002; Vaezi et al., 2002). Biochemical and genetic analyses of Lar indicate a role in regulating the actin cytoskeleton. Loss of Lar function in Drosophila oocytes results in defects in follicle formation, egg elongation and anterior-posterior polarity that are correlated with defects in actin filament organization (Bateman et al., 2001; Friedman and Spradling, 2001). Lar might help to maintain hub-GSC adhesion by interacting with and modulating the function of regulators of F-actin. Drosophila Lar and its homologs physically and genetically interact with Ena, a member of the Ena/VASP family of actin regulators (Biswas et al., 2002; Wills et al., 1999). Drosophila Ena and its mammalian homologs localize to adherens junctions and have been implicated in the formation and strengthening of adherens junctions in several cell types (Baum and Perrimon, 2001; Grevengoed et al., 2001; Kris et al., 2008; Scott et al., 2006; Vasioukhin et al., 2000). However, although Ena localized to the hub-GSC interface, where adherens junctions are present, its function was not absolutely required for GSC maintenance, suggesting that other F-actin regulators in addition to Ena function to maintain hub-GSC adhesion.

Lar protein localized to the hub-GSC interface might instead, or in addition, regulate the tyrosine phosphorylation state of components of the adherens junctions to maintain strong adhesion between hub cells and GSCs. Regulation of the tyrosine phosphorylation of components of adherens junctions plays an important role in modulating the adhesive state of cells (Daniel and Reynolds, 1997; Lampugnani et al., 1997; Roura et al., 1999; Wang and Hartenstein, 2006). Tyrosine phosphorylation of E-cadherin in epithelial cells induces loss of cell-cell contacts and the endocytosis of E-cadherin-GFP expressed in GSCs and early germ cells using the NGVP16 driver detected with anti-GFP (green, A; white, A’; C’) in 0- to 1-day-old wild-type (A,A’), Lar2127+/+ (B,B’) or Lar2127/Lar2127 (C,C’) mutant adult Drosophila testes. Vasa (red) marks germ cells and Arm (magenta) marks the hub. Arrowheads (C,C’) mark the interface between hub and a Lar2127/Lar2127 mutant GSC where E-cadherin-GFP fails to localize. (D-E’) Localization of E-cadherin-GFP (green, D,E; white, D’,E’) in homozygous Lar2127 mutant (arrows and white outline, D-D’) and Lar2127 mutant (arrows and white outline, E-E’) GSC clones 5 days PCI as compared with heterozygous control GSCs (arrowheads and red outline, D’,E’). Homozygous mutant GSCs are detected by the absence of anti-β-galactosidase immunostaining (red, D,E; white, D’,E’). (D’,E’) There is reduced or absent E-cadherin-GFP at the hub-GSC interface in homozygous mutant GSCs (arrows), whereas localization of E-cadherin-GFP is seen at the hub-GSC interface in heterozygous GSCs (arrowheads). The star indicates the Hub. (F) Quantitation of GSCs with E-cadherin-GFP expression and localization at the hub-GSC interface versus GSCs with little or no E-cadherin-GFP expression as detected in heterozygous and homozygous Lar2127 GSCs from clonal analysis. N, number of GSCs scored for each genotype. P-values of Fisher’s exact test (two-tailed) are shown. Scale bars: 25 μm.
of E-cadherin (Fujita et al., 2002; Potter et al., 2005). A possible role of Lar is to maintain adherens junctions by dephosphorylating E-cadherin. Alternatively, Lar might target E-cadherin to the membrane to build adherens junctions, as has been shown for the mammalian homolog of Lar in cultured hippocampal neurons, where it promotes the accumulation of cadherin-catenin complexes at the synapse to enhance cell adhesion (Duñah et al., 2005). Alternatively, or in addition, Lar might regulate tyrosine phosphorylation of the catepins associated with E-cadherin at the hub-GSC interface. Tyrosine phosphorylation of β-catenin leads to loss of cadherin-β-catenin interaction and to internalization of E-cadherin, reducing the strength of adherens junctions (Lilien and Balsamo, 2005). Mammalian Lar has been shown to dephosphorylate β-catenin in vitro (Duñah et al., 2005; Kypka et al., 1996; Muller et al., 1999), suggesting that in vivo Lar might promote cell adhesion by regulating the phosphorylation of β-catenin.

In addition to GSCs, Lar protein was also detected in two-, four- and eight-cell-stage amplifying spermatogonial cysts, which have the ability to dedifferentiate and reoccupy the hub to replace lost GSCs (Brawley and Matunis, 2004; Cheng et al., 2008; Sheng et al., 2009). Under conditions that promote dedifferentiation, spermatogonial cells send out dynamic, actin-rich, thin protrusions, suggesting acquisition of motility (Sheng et al., 2009). An intriguing possibility is that Lar might facilitate the ability of dedifferentiating spermatogonial cells to reorganize their actin morphology and function at synapses (Johnson et al., 2006).

Acknowledgements
We thank the Bloomington Drosophila Stock Center and Drosophila Genomics Research Center for fly stocks; the Drosophila Studies Hybridoma Bank for providing antibodies; Thomas Clandinin, Dorothea Godt; Ruth Lehmann, Mariann Bienz, Steven Hou and Erieh Bach for generously providing reagents; Thomas Clandinin and members of the Fuller laboratory for helpful discussions and advice; and Leanne Jones and Erin Davies for sharing microarray data in which Lar expression was identified in early germ and cyst cells.

Funding
This work was supported by the National Institutes of Health (National Research Service Award (NRSA) Postdoctoral Fellowship F32 GM083351 to S.S., NIH R01 GM080591 to M.T.F.). M.T.F. gratefully acknowledges the Reed-Hodgson Professorship in Human Biology, which partially supported this work. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.0700522-DJC1

References


