The receptor tyrosine phosphatase Lar regulates adhesion between *Drosophila* male germline stem cells and the niche

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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. *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, *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 Wagers, 2008; Kiger et al., 2001; 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 (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 divisions, allowing for either asymmetric divisions 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 (Yamashita et al., 2003; Yamashita et al., 2007). 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 wFw 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 Lar51, FRT40A/CyO, P[Ubi-GFP,S65T], an allele with an uncharacterized molecular lesion [gift from T. Clandinin (Clandinin et al., 2001)]; (2) w Lar2127, FRT40A/CyO, 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) y w hs-flp122, FRTG13/Lar w, FRT40A/hs-flp122, a deficiency that deletes the 5′ portion of the lar gene [Bloomington Drosophila Stock Center (BDSC)]; and the following stocks carrying ena alleles: (1) w FRTG13, ena210/CyO and (2) w FRTG13, ena235/CyO; both alleles harbor point mutations in domains crucial for Ena function (Ahern-Djamali et al., 1998). The stocks (1) y w, hs-FLP22; FRT40A, Ubi-GFP/CyO, (2) y w, hs-FLP22; FRT40A, arm-lacZ/CyO and (3) y w, hs-FLP22; FRT40A, Ubi-GFP/CyO (BDSC) were paired with the above stocks for inducing mutant clones or paired with FRT40A/CyO or FRTG13/CyO (BDSC) for inducing wild-type control clones. nanos-GAL4-VP16 (NGVP16) (gift from R. Lehmann, Skirball Institute, NY, USA) (Van Doren et al., 1998) and CS877-GAL4 (gift from S. Hou, NCI, Frederick, MD, USA) drivers were used to express UAS-GFP-α-Tubulin in early germ cells and cyst cells, respectively. To drive expression of UAS transgenes in wild-type or Lar mutant early germ cells, scco/CyO; NGVP16 (BDSC) or Lar2127, FRT40A/CyO, NGVP16 virgin females were crossed to Lar51, FRT40A/CyO; UAS-DEFL -6-1. The UAS-DEFL -6-1 line [containing E-cadherin-GFP downstream of UAS sequences (Oda and Tsukita, 1999)] was acquired from the Drosophila Genomics Research Center, Kyoto, Japan.

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., 1992)), Enabled [rabbit, 1:5000; a gift from R. Lehmann] and Sat92E (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 spectrosome (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 Flipase (FLP)-mediated mitotic recombination. y w; hs-flp122, FRT40A or w, Lar51, FRT40A/CyO or w; Lar2127, FRT40A/CyO males. Similarly, y w; hs-flp122, FRTG13, Ubi-GFP virgin females were crossed to w; FRTG13 or w; ena235/CyO or w; ena210, FRTG13/CyO males to generate homozygous ena mutant clones. To compare E-cadherin-GFP localization in GSCs that were heterozygous and homozygous mutant for Lar function, hs-FLP22; FRT40A, arm-lacZ/CyO; NGVP16 virgins were crossed to either w; Lar51, FRT40A/CyO; UAS-DEFL -6-1 or w; Lar2127, FRT40A/CyO; 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 ena 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 dehydrated in ethanol and embedded in Epon resin. Serial sections (80 nm) were cut 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)E53 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.
By day 15 PCI, 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’), but not in the mutant (B’). Lar
tt/Lar
tt mutant (D,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) Germineal clonal analysis of the Lar mutant alleles Lar
tt (red) and Lar
tt (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 (PCI). Numbers next to the data points indicate the number of testes scored for each genotype at the different time points. The same fonts of numbers were scored for F and H. Data are average ± s.d. Scale bars: 25 μm.

Fig. 2. Lar functions cell-autonomously to maintain GSCs at the hub. (A-B’) Apical tips of 0–1-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) Lar
tt/Lar
tt 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’) Wild-type (C,C’) and Lar
tt/Lar
tt mutant (D,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) Germineal clonal analysis of the Lar mutant alleles Lar
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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 Lar
tt/Lar
tt adult testes had Stat92E protein in the cytoplasm and the nucleus (Fig. 4B,B’). Stat92E protein was also detected in

By day 15 PCI, 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 Lar
tt/Lar
tt mutant GSCS revealed that Ena failed to localize to the hub-GSC interface in about half the GSCs (arrowhead, Fig. 3B’). Of the 145 Lar
tt/Lar
tt 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 ena
tt and ena
tt were generated using the FLP-FRT system and scored for maintenance at different time points PCI. At 2 days PCI, 83.5% of testes carrying the ena
tt allele and 90% of testes carrying the ena
tt allele had at least one marked homozygous mutant GSC, comparable to wild-type controls (100% testes) (Fig. 3C). The percentage of testes with ena
tt homozygous mutant GSC clones dropped from 90% at day 2 PCI to 56% at day 3 and to 45.5% by day 15 PCI, 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 ena
tt did not drop significantly over time, remaining at 73.5% at day 15 PCI. 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.

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tt 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 Lar451/Lar2127 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 Lar451 or Lar2127 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 Lar451/Lar2127 testes, the Apc2 detected was distributed around the GSC cortex, rather than localized to the hub-GSC interface (Fig. 5B,B',E). 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 homozygous Lar451 and 57% of homozygous Lar2127 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; white C,D) were detected by the absence of anti-GFP immunostaining (green, C,D; white C,D). The star indicates the Hub. Scale bars: 25 μm.

Mislocalization of Apc2 in Lar mutant GSCs remaining at the hub might lead to an increased frequency of misoriented centrosomes in those cells. GSCs with two centrosomes in Lar mutant testes (Fig. 5G, supplementary material Fig. S2A,B) or in homozygous Lar mutant GSC clones induced in heterozygous animals (Fig. 5H) showed an increased percentage of misoriented centrosomes compared with their controls. In 0- to 1-day-old Lar451/Lar2127 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 Lar451 and Lar2127 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; white C,D) were 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 Lar451/Lar2127 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 (Fig. 5A,A'). Immunostaining with anti-Apc2 revealed that, in 57.5% of the GSCs that remained adjacent to the hub in Lar451/Lar2127 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 Lar451 or Lar2127 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.

**Fig. 4. Lar function is not required for Stat92E accumulation in GSCs.** (A-B') 0- to 1-day-old wild-type (A,A') and Lar451/Lar2127 mutant (B,B') adult Drosophila testes immunostained with anti-Stat92E (red, A,B; white, A',B') and anti-Vasa (green) antibodies. (A,B) GSCs (arrows) and CySCs (arrowheads) express Stat92E. (B') A germ cell (long arrow) displaced from the hub expresses Stat92E. (C-D') Expression of Stat92E (red, C,D; white, C',D') in homozygous Lar451 (arrows, C,C') and Lar2127 (arrows, D,D') mutant GSC clones 5 days PCI as compared with heterozygous control GSCs (arrowheads). Homozygous mutant GSCs (green outline in C,D; white 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.
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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 of the hub-GSC interface were juxtaposed at the hub-GSC interface, 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 Lar451/Lar2127 mutant testes (two-tailed) are shown. Scale bars: 25 μm.

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 (supplementary material Fig. S3A, A’), was reduced in Lar451/Lar2127 mutant testes (supplementary material Fig. S3A, A’), in Lar451/Lar2127 mutant testes and (H) in heterozygous and homozygous FRT40A, Lar451 and Lar2127 GSCs. N, number of GSCs scored for each genotype. P-values of Fisher’s exact test (two-tailed) are shown. Scale bars: 25 μm.
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-E-cadherin-GFP specifically in the germ cells under the control of the NGVP16 driver. E-cadherin-GFP was expressed in wild-type (C,C’) 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 Lar mutants, Lar function appears to promote the maintenance of robust adherens junctions between GSCs and hub cells and to localize and/or retain E-cadherin at the hub-GSC interface. Consistent with the recently demonstrated requirement for E-cadherin to polarize GSCs by localizing Apc2 at the hub-GSC interface and to establish centrosome orientation in GSCs (Inaba et al., 2010), Apc2 was often mislocalized around the GSC cortex and centrosomes were often misoriented in Lar mutant GSCs.

Lar may function in parallel with other cell signaling pathways that are important for maintaining attachment of GSCs to the hub. Activation of the JAK-STAT pathway in Drosophila male GSCs at the hub (Leatherman and Dinardo, 2010). However, Stat92E protein levels appeared normal in Lar mutant GSCs, suggesting that Lar function is not required for activation of the JAK-STAT pathway. The Rap1 GTPase/Rap1 guanine nucleotide exchange factor (Rap-GEF) signaling pathway also regulates hub-GSC adhesion (Wang et al., 2006). Like Lar mutants, Rap-GEF mutants have impaired adherens junctions at the hub-GSC interface resulting in GSC loss. However, Rap-GEF function is required in hub cells, whereas Lar functions in GSCs to promote hub-GSC adhesion. Interestingly, expression of E-cadherin-GFP in either hub cells or GSCs of Rap-GEF mutants resulted in wild-type numbers of GSCs and restored E-cadherin localization at the hub-GSC interface (Wang et al., 2006), whereas expression of E-cadherin-GFP in Lar mutant GSCs did not rescue the loss of GSCs, suggesting that the Rap-GEF and Lar signaling pathways might use different mechanisms to build and/or maintain adherens junctions between the hub cells and GSCs.

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,
concentration of cortical F-actin (Hirokawa et al., 1983), organized by the high local 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 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 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 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, 2000). 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; Frydman 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 (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 (Dunah et al., 2005). Alternatively, or in addition, Lar might regulate tyrosine phosphorylation of 

Dally-like (Dlp), is expressed by hub cells and helps of the actin cytoskeleton (Chagnon et al., 2004). One of the ligands hub, similar to the role of Lar in the nervous system, where it 

suggesting acquisition of motility (Sheng et al., 2009). An 

inhibition of the phosphatase activity of Lar to regulate active zone 


Mutations in Drosophila enabled and rescue by human vasodilator-stimulated phosphoprotein (VASP) indicate important functional roles for Ena/VASP homology domain 1 (EVH1) and EVH2 domains. Mol. Biol. Cell 9, 2157-2171.


