



Dynein light chain 1 functions in somatic cyst cells regulate spermatogonial divisions in *Drosophila*

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Stem cell progeny often undergo transit amplifying divisions before differentiation. In *Drosophila*, a spermatogonial precursor divides four times within an enclosure formed by two somatic-origin cyst cells, before differentiating into spermatocytes. Although germline and cyst cell-intrinsic factors are known to regulate these divisions, the mechanistic details are unclear. Here, we show that loss of *dynein-light-chain-1* (DDLC1/LC8) in the cyst cells eliminates *bag-of-marbles* (*bam*) expression in spermatogonia, causing gonial cell hyperplasia in *Drosophila* testis. The phenotype is dominantly enhanced by *Dhc64C* (cytoplasmic Dynein) and *didum* (*Myosin V*) loss-of-function alleles. Loss of DDLC1 or *Myosin V* in the cyst cells also affects their differentiation. Furthermore, cyst cell-specific loss of *ddlc1* disrupts Armadillo, DE-cadherin and Integrin- β PS localizations in the cyst. Together, these results suggest that Dynein and *Myosin V* activities, and independent DDLC1 functions in the cyst cells organize the somatic microenvironment that regulates spermatogonial proliferation and differentiation.

Stem cells have sustained self-renewal ability, and their progeny differentiate into tissue-specific cell types. Most stem cell progeny undergo a limited number of divisions (transit amplification) before terminal differentiation¹, which is tightly regulated to maintain tissue homeostasis and prevent cancerous growth. Regulatory mechanisms underlying the tight control of transit amplification are still unclear. However, it is evident that the local microenvironment plays a vital role in tumor formation and metastatic growth^{2,3}. Spermatogenesis in *Drosophila* is a powerful model for genetic dissection of stem cell biology⁴⁻⁶. *Drosophila* testis harbors two groups of stem cells, the germline stem cells (GSCs) and the somatic stem cells (SSCs)⁷, anchored to a group of somatic cells (hub) at the apical end. Spermatogenesis begins with an asymmetric division of a GSC into two cells, one of which retains its stem cell properties by remaining in contact with the hub whereas the other begins differentiation as a gonialblast that undergoes four mitotic divisions before differentiating into spermatocytes. The two SSCs, juxtaposed to a GSC, also divide to form two cyst cells that enclose the gonialblast and its progeny. The cyst cells do not divide. They maintain an intimate contact with the germ cells throughout spermatogenesis⁴. Both germ cell intrinsic factors and signaling processes in the supporting cyst cells are essential for precise control of spermatogonial divisions and differentiation.

Previous studies showed that *bag-of-marbles* (*bam*) and *benign-gonial-cell-neoplasm* (*bgn*) act in a cell-autonomous manner to regulate spermatogonial divisions⁸. Bam functions as a translational repressor of *nanos* mRNA in the female germline⁹, and its expression in the male germline coincides with the commencement of spermatogonial differentiation¹⁰. Progressive accumulation of the Bam protein in dividing spermatogonia provides a counting mechanism that controls the number of divisions¹¹. In addition, disruption of TGF β ^{12,13}, D-raf⁶, and EGFR¹⁴ pathways in the somatic cyst cells upregulate germ cell divisions and block further differentiation. Expression of Spitz, an EGF-like ligand, in the germ cells initiates the EGFR signaling in the cyst cells by activating Rac1-GTPase through a guanine nucleotide exchange factor Vav inside the cyst cell¹⁵. The identity and source of the ligands that activate the TGF β pathway in the somatic cyst cells, however, are not well understood¹⁶. Also, it is unclear how various signaling pathways interact within the cyst cells and give feedback to the germline to limit the transit amplification of spermatogonia.

Motor proteins such as Kinesins and cytoplasmic Dynein mediate signal transduction by transporting components of signaling pathways¹⁷⁻²⁰ and, therefore, could potentially mediate downstream interactions between these pathways. Indeed, an increase in the cellular levels of Dynein light chain 1 (DLC1/LC8), the 8 kDa



conserved light chain of cytoplasmic Dynein, and a missense mutation in DLC1 are both associated with cell proliferation in certain types of cancers^{21,22}. A recent study in *C. elegans* also showed that ubiquitous loss of DLC1 and Dynein functions cause excessive germ cell proliferation²³, indicating a distinct role of these molecules in germline homeostasis. However, it is uncertain whether this phenotype is caused due to the loss of DLC1-dependent cytoplasmic Dynein activity in the germ cells. This is because loss of mitotic function of Dynein would be expected to inhibit germ cell division. Moreover, DLC1 is not required for the Dynein function in mitosis²⁴. Therefore, the nature of DLC1 and Dynein functions in regulation of germ cell divisions is unclear.

We had earlier shown that mutations in the *Drosophila dynein light chain-1* (*ddlc1*) gene causes male sterility, and DDLC1 is required in the germ cells for spermatid elongation and individualization^{25,26}. Here, we find that a partial loss of DDLC1 in early cyst cells, but not in germ cells, deregulates the cell cycle-arrest in spermatogonia, and produces a neoplastic germline in aged adults. Our analysis further indicates that along with the cytoplasmic Dynein, the activities of Myosin V (*didum*), a motor involved in membrane recycling and secretion^{27,28}, and Rab11, one of the key components of membrane recycling cargoes of Myosin V²⁹, in the somatic cyst cells regulate germ cell division and differentiation. These unexpected findings suggest that specific motor-based signaling processes within the somatic cyst cells are involved in processing the feedback signal required for regulation of germ cell divisions.

Results

Hypomorphic mutations in the *ddlc1* gene deregulate transit amplifying divisions and affect spermatogonial differentiation.

A preliminary investigation with *ddlc1* mutants indicated an increase in the number of mitotic cells at the apical region of testis. Therefore, we explored it further by comparing the expression of early germline and cyst cell-specific markers in four-day-old Canton-S (wild type) and partial-loss-of-function *ddlc1^{ins1}* hemizygous testes (Figure 1A). The small spermatogonial cells (arrowhead, Figure 1A-a) and the larger spermatocytes (arrow, Figure 1A-a) are labeled with Vasa, an exclusive germline marker³⁰. The *ddlc1^{ins1}* testes contained only brightly labeled, small Vasa-positive cells, resembling the early stage spermatogonia (arrowheads, Figure 1A-b). In addition, the branched tubular fusome, usually found in the differentiating spermatogonia³¹ (arrow, Figure 1A-c), appeared comparatively thinner and less branched (arrow, Figure 1A-d) in the *ddlc1^{ins1}* testes. Together, these two observations suggested that the loss of DDLC1 arrests spermatogonial differentiation. The *UAS-eGFP* expression due to *nosGal4* marks the germline stem cells and the early spermatogonial precursors in wild type testes (arrow, Figure 1A-e), and the *saCD8GFP*³² depicts the *spermatocyte arrest* expression in differentiated spermatocytes (arrow, Figure 1A-g). In *ddlc1^{ins1}; nosGal4/ UAS-eGFP* testis, the GFP fluorescence marked almost all the cells in the apical region (arrow, Figure 1A-f), whereas the *saCD8GFP* expression was totally abolished in the *ddlc1^{ins1}; saCD8GFP/+* testis (Figure 1A-h). The extended *nanos* promoter activity, depicted by the *nosGal4* expression, and subsequent loss of the *saCD8GFP* expression established that spermatogonial differentiation to spermatocytes is arrested in the *ddlc1^{ins1}* mutant testis.

The transition from spermatogonia to spermatocytes is synchronized with the cyst cell differentiation^{33,34}. It is indicated by the Traffic Jam (TJ) expression in the early cyst cell nuclei (arrow, Figure 1A-i), and Eyes absent (*Eya*) induction (arrows, Figure 1A-k) in the subsequent stages (Supplementary Figure S1, I-A). The suppression of TJ expression in the cyst cells coincides with the commencement of the *saCD8GFP* expression in the spermatocytes within the cyst (Supplementary Figure S1, I-B). The anti-TJ staining persisted in the cyst cells (arrows, Figure 1A-j), whereas the anti-*Eya*

staining was absent (Figure 1A-l) in *ddlc1^{ins1}* mutant testes. This showed that the loss of *ddlc1* also arrests the cyst cell differentiation.

The nuclei of proliferating GSCs, gonialblasts and spermatogonia, found at the apical region of a wild-type testis, are strongly labeled with the DNA-specific dye, DAPI (arrow, Figure 1B-a). This staining was vastly expanded in 3 days old *ddlc1^{ins1}* and *ddlc1^{DIIA82}* hemizygous testes (arrows, Figures 1B-b, c), and continued to expand with aging (Supplementary Figure S1-II). We reasoned that increased mitotic division in the tissue or an arrest of spermatocyte differentiation could cause such a phenotype. To distinguish between these two possibilities, testes from four-day-old wild-type, *ddlc1^{ins1}* and *ddlc1^{DIIA82}* flies were pulse-labeled with 5-bromo-2-deoxyuridine (BrdU) for one hour. The BrdU label marked a few clusters of spermatogonia and stem cell nuclei at the apex of the wild-type testis (arrow, Figure 1B-d). The label, however, was widespread in the *ddlc1* mutant testes (arrows, Figures 1B-e, f). The BrdU-incorporation in the chromatin serves as a marker of the S-phase of cell cycle³⁵. Therefore, this demonstrated that the expanded gonial cell population actively proliferates in the *ddlc1* mutants. Further analysis of Vasa, TJ and phospho-Histone H3 (PH3) staining of *ddlc1* mutant testes (Figures 1B-h, i) confirmed that the higher mitotic activity in the mutant testes is limited to the germ cells (Figure 1B-j). In addition, a 5-minute BrdU pulse-labeling of two and three-days old wild-type and *ddlc1^{ins1}* testes showed that the ectopic spermatogonial proliferation in *ddlc1^{ins1}* testes mostly starts between two and three days after eclosion (Figure 1B-l, m). This suggested an age-dependent increase in the proliferating potential of the mutant spermatogonia. Hemizygous *ddlc1^{ins1}* males are sterile²⁶, and the phenotypes described above are fully penetrant by day 4 after the eclosion at 29°C. The *ddlc1* mRNA level is comparatively lower in the *ddlc1^{DIIA82}* allele²⁶, and the phenotypes were accordingly more severe in the *ddlc1^{DIIA82}* hemizygous testes. Thus, we concluded that partial loss of *ddlc1* causes germ cell hyperplasia in the testes of aged adults.

DDLC1 functions in the cyst cells regulate transit amplifying divisions of spermatogonia.

Both germ cell-intrinsic and cyst cell-derived signals control spermatogonial proliferation^{6,8,14}. DDLC1 is ubiquitously expressed (Supplementary Figure S2-IV). Therefore, disruption of DDLC1-dependent cellular functions in either germline or soma could result in germ cell hyper-proliferation in *ddlc1* mutant testis. The rescue of this phenotype with tissue-specific expression of *ddlc1* transgenes (*UAS-ddlc1* and *UAS-mycPIN*) in *ddlc1* mutant background resolved this issue. *nosGal4* drives gene expression in the germline whereas *ptcGal4* expresses in the somatic cells¹⁰ (Supplementary Figure S2-I). The BrdU incorporation levels in *ddlc1^{ins1}; UAS-ddlc1/+*; *nosGal4/+* and *ddlc1^{ins1}; UAS-mycPIN/+*; *nosGal4/+* testes were similar to that in the *ddlc1^{ins1}* and *ddlc1^{ins1}; UAS-mycPIN/+* controls (Figures 2A-a, b, e). It was, however, comparable to wild-type levels in the *ddlc1^{ins1}; UAS-mycPIN/ptcGal4* and *ddlc1^{ins1}; UAS-ddlc1/ptcGal4* testes (Figures 2A-c, e). Additionally, high levels of BrdU incorporation were also observed in the *ptcGal4/+*; *UAS-ddlc1^{dsRNA}/+* testis (Figures 2A-d, e), suggesting that dsRNA-mediated knockdown of *ddlc1* in somatic cells could also cause excessive germ cell proliferation. These results clearly showed that DDLC1 function is essential in the somatic cells for controlling the spermatogonial proliferation.

To identify the somatic cells in which *ddlc1* is required for controlling spermatogonial proliferation, we crossed *UAS-ddlc1^{dsRNA}* to different somatic cell type-specific *Gal4* drivers (Figure 2B). The *UAS-GFP* expression due to *tjGal4*^{34,36} marked the hub, SSCs and early cyst cells (Figure 2B-a), whereas the expression of the same reporter gene due to *updGal4*³⁷ exclusively marked the hub (Figure 2B-b). The *esgGal4*-dependent *UAS-GFP* expression marked the SSCs and the early stage cyst cells (Figure 2B-c). In addition, we found that the expression due to *daGal4*³⁸, which marked the early stage cyst cells (arrowheads, Figure 2B-d) and the spermatocytes

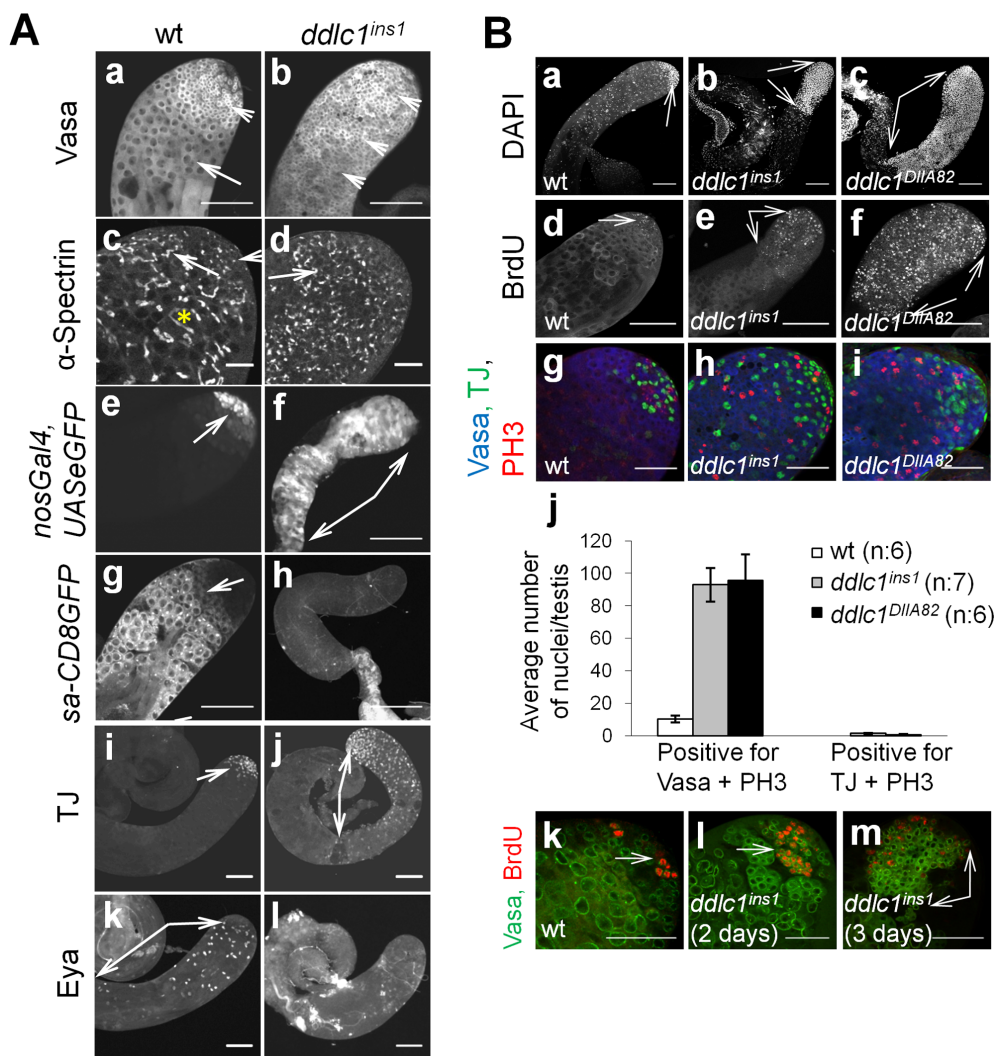


Figure 1 | Cell differentiation and proliferation defects in *ddlc1^{ins1}* hemizygous mutant testes. (A) Wild type (a, c, e, g, i, k) and *ddlc1^{ins1}* (b, d, f, h, j, l) hemizygous testes stained with antibodies against Vasa (a, b), α -Spectrin (c, d), Traffic Jam (i, j) and Eyes absent (k, l), or, marked by *nosGal4*; *UAS-eGFP* (e, f) and *sa-CD8GFP* (g, h) expression, indicate the state of germline differentiation. (a, b) Arrowheads indicate location of spermatogonia and arrow points to spermatocytes. (c, d) Arrowhead points to a spectrosome and arrows point to branched fusomes. Yellow asterisk indicates a spermatocyte fusome. (e, f) Arrows mark the early spermatogonial cells. (g, h) Arrow marks the onset of Sa-CD8GFP expression in the spermatocytes. (i–j) Arrows point to the Traffic Jam (TJ) positive nuclei of early stage cyst cells. (k, l) Arrow indicates the location of Eya-positive cyst cells. Scale bars (except for c, d): 100 μ m. Scale bars (c–d): 25 μ m. (B) Four-day-old wild type (a, d, g), *ddlc1^{ins1}* (b, e, h), and *ddlc1^{DIIA82}* (c, f, i) testes, labeled with one-hour BrdU pulse, and stained with DAPI (a–c) and anti-BrdU (d–f) are shown. Arrows depict the region intensely stained with DAPI and BrdU. (g–i) Wild type and *ddlc1* testes labeled with Vasa (blue), TJ (green), and phospho-Histone H3 (PH3, red) antibodies. (j) Average number of PH3 positive nuclei co-labeled with Vasa or TJ in wild type, *ddlc1^{ins1}*, and *ddlc1^{DIIA82}* testes. Error bars indicate \pm SEM. (k–m) Two-day-old wild type (k), and two (l) and three-day old (m) *ddlc1^{ins1}* testes were stained with anti-Vasa (green) and anti-BrdU (red) antibodies after a brief (5 minutes) BrdU pulse labeling. (k, l) arrows indicate individual BrdU labeled cysts. (m) Arrows indicate the region containing large cyst-like aggregates with few BrdU labeled nuclei. Scale bars (a–f): 100 μ m. Scale bars (g–i and k–m): 50 μ m. *ddlc1^{DIIA82}* is a relatively stronger hypomorph than *ddlc1^{ins1}* mutant²⁶.

(yellow asterisk, Figure 2B-d), excluded the 8-16 cell stage spermatogonial cysts (arrow, Figure 2B-d). Amongst the Gal4 drivers used, *tjGal4* expression was the strongest and continued in the cyst cells until the early spermatocyte stage.

The *UAS-ddlc1^{dsRNA}* expression driven by *tjGal4* caused excessive germ cell proliferation similar to that found in *ddlc1* mutant testes (Figure 2B-e). However, the *UAS-ddlc1^{dsRNA}* expression due to *updGal4* (Figure 2B-f), *esgGal4* (Figure 2B-g), and *daGal4* (Figure 2B-h) failed to induce this defect. The *esgGal4/+*; *UAS-ddlc1^{dsRNA}*/+ testis, however, contained relatively fewer spermatogonial cells and some early stage spermatocytes (Figure 2B-g). It lacked the other differentiated stages such as the elongated spermatids and the mature sperm bundles. Similarly, further differentiation of spermatocytes was arrested in the *UAS-ddlc1^{ins1}*/ *daGal4* testis. Thus, the

results suggest that a certain amount of *ddlc1^{dsRNA}*, as produced due to *tjGal4*, is required in the cyst cells during the spermatogonial stages to generate the hyperplasia. Consistent with this, both the somatic and germline differentiation defects were rescued in the *ddlc1^{ins1}*; *tjGal4/UAS-mycPIN* testis (Supplementary Figures S2-II, III). Taken together, these results indicated that DDLC1 functions in the cyst cells enclosing the proliferating spermatogonia regulate their divisions. Escargot (*esg*) also expresses in somatic cells of the developing gonad³⁹, and defines the stem cell niche (hub) as well as the number of GSCs in the mature testis⁴⁰. Although it did not cause hyperplasia, the *UAS-ddlc1^{dsRNA}* expression due to *esgGal4* reduced the overall pool of gonial tissue, which may occur due to a reduction in the number of GSCs. This could reflect another novel role of DDLC1 in the somatic tissue of the early gonad.

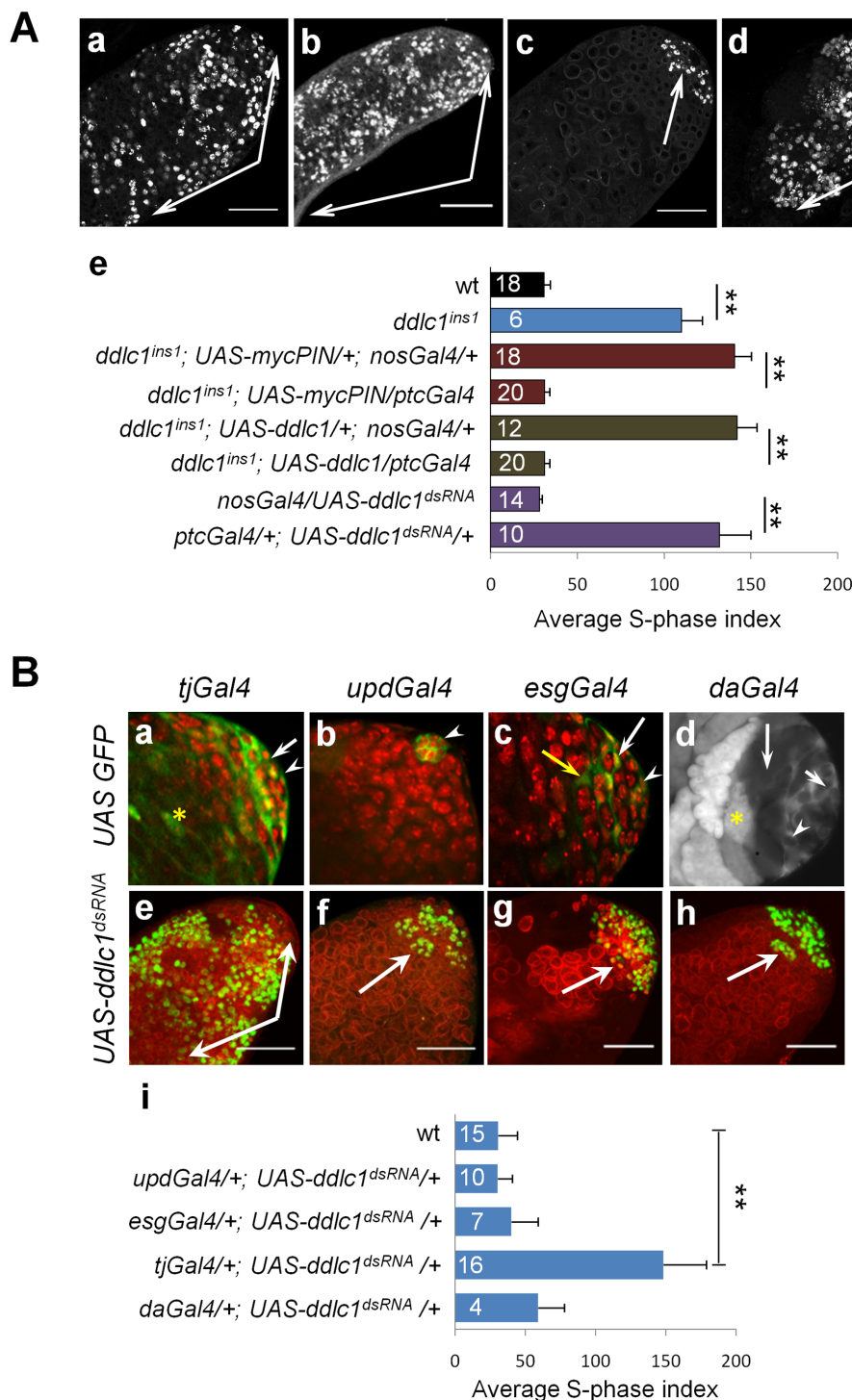


Figure 2 | Tissue-specific requirement of *ddlc1* during the transit amplification of spermatogonia. (A) Testes from four-day-old *ddlc1^{ins1}; UAS mycPIN/+* (a), *ddlc1^{ins1}; UAS mycPIN/+; nosGal4/+* (b), *ddlc1^{ins1}; UAS mycPIN/ptcGal4* (c) and *ptcGal4/+; UAS-ddlc1^{dsRNA}/+* (d) adults were pulse-labeled with BrdU for one hour before anti-BrdU staining. Arrows indicate regions containing BrdU positive nuclei. (e) Histograms show average S-phase indices (average number of BrdU positive nuclei per testes) at four days post-eclosion from different genetic backgrounds (mentioned on y-axis). The number of samples used for each genotype is indicated on the bars. Data are shown as mean \pm SEM. (B) *UAS-GFP* expression due to the *tjGal4* (a), *updGal4* (b) and *esgGal4* (c) variedly marked the hub (arrowhead), somatic stem cells (arrows), and the early (yellow arrow) and late cyst (yellow asterisk) cells. The GFP expression due to the *daGal4* (d) labeled the somatic cyst cells until the 4-cell spermatogonia stage (arrowheads), excluded the 8–16 cell cysts (arrow), and resumed in the early spermatocytes (yellow asterisk). Testes from four-day-old *tjGal4/+; UAS-ddlc1^{dsRNA}/+* (e), *updGal4/+; UAS-ddlc1^{dsRNA}/+* (f), *esgGal4/+; UAS-ddlc1^{dsRNA}/+* (g), and *daGal4/UAS-ddlc1^{dsRNA}/+* (h) flies were stained with anti-Vasa (red) and anti-BrdU (green) antibodies after one-hour BrdU pulse-labeling. (i) Average S-phase indices resulting from *ddlc1^{dsRNA}* knockdown in different subsets of somatic cells in the testis are plotted. The genotypes used are depicted on the left margins of the plot. The number of samples used for each genotype is indicated on the bars. Data are plotted as mean \pm SEM. Scale bars indicate 50 μ m and ** indicates $p < 0.001$ obtained by the Mann-Whitney test.



Loss of *didum* (Myosin V) in cyst cells causes excessive germ cell proliferation. DLC1 plays a critical role in protein dimerization⁴¹. Two of its confirmed interacting partners are the IC74 subunit of the cytoplasmic Dynein complex⁴² and Myosin V. In addition, it interacts with many other cellular proteins^{43–47}. Therefore, amongst several other reasons, the proliferation and differentiation defects observed in the *ddlc1* mutant testis could be attributed to the loss of Dynein or Myosin V functions, or both, in the cyst cells. The cyst cell-specific expression of the *UAS-Dhc64C^{dsRNA}* and *UAS-*didum*^{dsRNA}* resulted in spermatogonial hyperproliferation (Figure 3A). The average S-phase indices were similar in testes expressing either *ddlc1* or *didum* dsRNAs, and comparatively less in the testis expressing the *Dhc64C* dsRNA (Figure 3B). Such an effect could occur due to a difference in the efficacy of the dsRNA reagents. In addition, the S-phase indices measured two days after eclosion were significantly enhanced in the *ddlc1^{ins1}*; *Dhc64C^{6-10/+}* and *ddlc1^{ins1}*; *didum^{KG04384/+}* testes as compared to that in the *ddlc1^{ins1}* testis (Figure 3B). Both the *Dhc64C⁶⁻¹⁰* and *didum^{KG04384}* are null alleles and the enhancement of the cell proliferation defect was marginally stronger in the *ddlc1^{ins1}*; *didum^{KG04384/+}* testis than that in the *ddlc1^{ins1}*; *Dhc64C^{6-10/+}* testis. Therefore, these two results suggested that along with DDLC1, cytoplasmic Dynein and Myosin V functions in the cyst cells play a key role in regulating spermatogonial proliferation.

Loss of DDLC1 in cyst cells eliminates *bam* expression in spermatogonia. The transgenic rescue of the *ddlc1* phenotype as well as the *ddlc1* knockdown study in the cyst cells indicated that DDLC1 activity is most critical in the cyst cells encapsulating the 4–16 cell spermatogonial cysts. This coincides with the onset of *bam* expression in the germline^{8,13}, and the accumulation of Bam to a critical level arrests spermatogonial proliferation¹¹. Bam is also responsible for the translational repression of *nanos* mRNA in the cystoblast cells in the ovary⁹. Hence, extended *nosGal4*, *UAS-eGFP* expression observed in the *ddlc1^{ins1}* testis could be coupled to Bam repression. Indeed, the Bam immunostaining disappeared in the *ddlc1^{ins1}* testis between two and three-days after eclosion (Figure 4A–b, c; Supplementary Figure S3), and the homologous *mycPIN* transgene expression in the early cyst cells rescued the defect in the *ddlc1^{ins1}*; *tjGal4/UAS-mycPIN* testis (Figure 4A–d). Together, these results suggested that DDLC1 functions in the cyst cells maintain Bam levels in the germline.

To understand whether this phenomenon is caused due to decreased response of *bam* promoter or loss of Bam protein stability in the *ddlc1* mutant, we monitored *bamP-GFP* (*bmP⁷⁰²-GFP*)⁴⁸ and *bamP-bam::GFP*⁴⁹ expressions in *ddlc1^{ins1}* background (Figure 4B and C). The former reports the *bam* promoter activity whereas the latter expresses Bam::GFP fusion protein under the *bam* promoter^{48,49}. The *bamP-GFP* label visibly declined with aging, and it

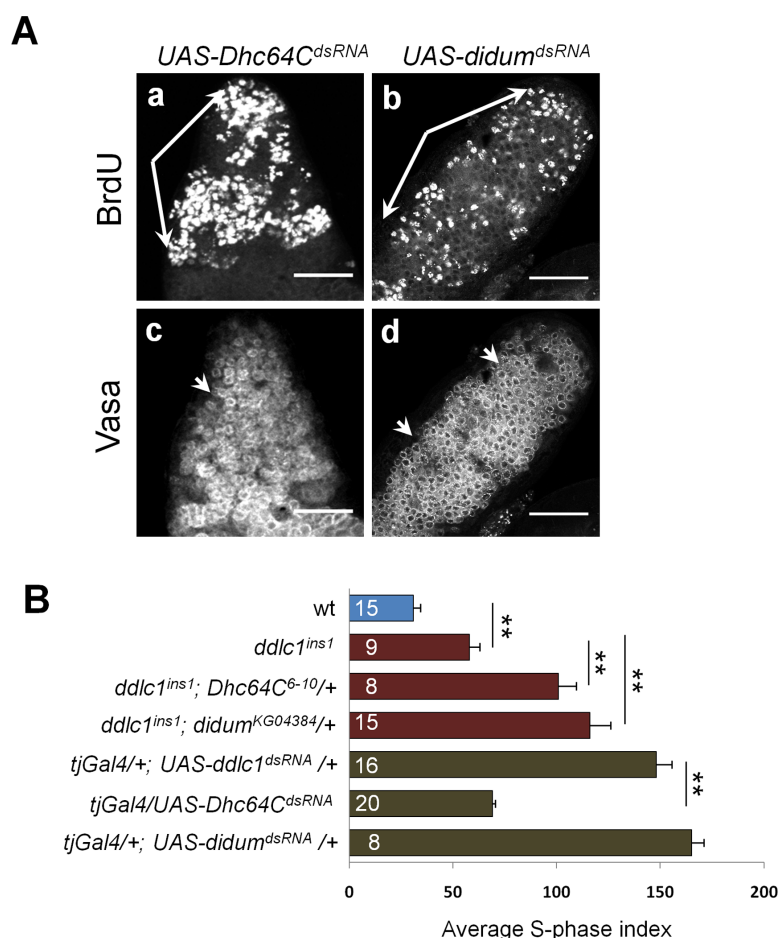


Figure 3 | Excessive germline proliferation due to cyst cell-specific knockdown of *Dhc64C* and *didum* (myosin V). (A) Four-day old *tjGal4/UAS-Dhc64C^{dsRNA}* (a, c) and *tjGal4/UAS-didum^{dsRNA}* (b, d) testes were pulse-labeled with BrdU for one hour and stained with anti-BrdU (a, b) and anti-Vasa (c, d) antibodies. Arrows in (a, b) indicate regions containing BrdU positive nuclei, and arrowheads in (c, d) indicate small-sized spermatogonia. (B) Histograms depict average S-phase indices from various genotypes (indicated at the left margin) after a one-hour BrdU-pulse labeling. The number of samples used for each genotype is indicated on the bars. Data are plotted as mean \pm SEM. Scale bars indicate 50 μ m in all Figures and ** indicates $p < 0.001$ obtained by the Mann-Whitney test.

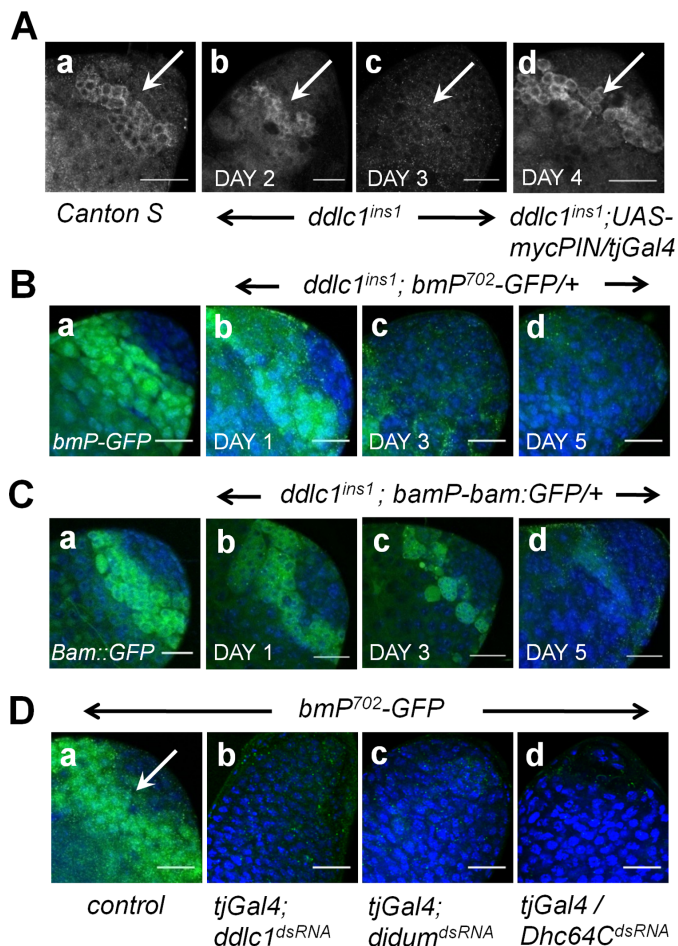


Figure 4 | Analysis of Bam expression in the *ddlcl^{ins1}* testes. (A) Testes from four-day old wild type (a) and *ddlcl^{ins1}; UAS-mycPIN/tjGal4* (d) as well as two (b) and three days old (c) *ddlcl^{ins1}* flies were stained with anti-Bam antibody (arrows). (B) *bamP-GFP* expression (green) in wild type (a) and *ddlcl^{ins1}* (b-d) testes, aged for one day (b), three days (c), and five days (d) after eclosion. (C) *BamP-Bam::GFP* expression (green) in wild type (a) and *ddlcl^{ins1}* (b-d) testes aged for one day (b), three days (c), and five days (d) after eclosion. (D) GFP expression (green) driven by *bam* promoter in testes from 4 days old *tjGal4/+; bamP⁷⁰²-GFP/+* (a), *tjGal4/+; UAS-ddlc1^{dsRNA}/bamP⁷⁰²-GFP* (b), *tjGal4/+; UAS-didum^{dsRNA}/bamP⁷⁰²-GFP* (c), and *tjGal4/UAS-Dhc64C^{dsRNA}/bamP⁷⁰²-GFP/+* (d) flies are shown. Nuclei in panels B, C, and D are stained with the Hoescht DNA labeling dye (blue). Scale bars in B, C and D represent 50 μm and scale bars in A represent 25 μm .

was fully repressed at three-days after eclosion (Figure 4B), whereas the Bam::GFP was visible in the spermatogonia until three-days after eclosion in the *ddlcl^{ins1}* testis (Figure 4C). The relatively longer persistence of Bam::GFP could be caused due to an over expression or a relatively higher stability of the recombinant protein. Expectedly, the cyst cell-specific expression of the transgenic mycPIN in *ddlcl^{ins1}* testis was sufficient to maintain the *bamP-GFP* expression in the mutant germline (Figure 4A-d, Supplementary Figure S3). In addition, the *bamP-GFP* expression was eliminated due to cyst cell-specific expression of *ddlcl* (Figure 4D-b), *didum* (Figure 4D-c) and *Dhc64C* (Figure 4D-d) dsRNAs. These results further suggested that, along with DDLC1, both Myosin V and Dynein functions are necessary in the early stage cyst cells to induce *bam* expression in the spermatogonia.

Rab11 knockdown in the cyst cells causes germ cell hyperplasia. Dynein mediates intracellular signaling via endosomal transport^{20,50,51}, whereas Myosin V is involved in membrane recycling and the apical

secretion events^{27,29}. Therefore, both the signal reception and transmission events in the somatic cyst cells could be involved in regulating signaling events between the germ cells and the cyst cells. Small G-proteins, belonging to Rab families, affect signal transduction through endosomal trafficking and Rab11 is shown to play an essential role in Myosin V-dependent apical secretion²⁹. We found that the *tjGal4/+; UAS-rab11^{dsRNA}/+* testes were unusually small (yellow circle, Figure 5a), and contained small Vasa-positive germ cells (arrow, Figure 5b) bearing spectrosomes (arrowhead, Figure 5c and inset c') or dumbbell-shaped fusomes (arrow, Figure 5c and inset c''). These cells incorporated BrdU upon one hour pulse-labeling (yellow circle, Figure 5d), indicating that they were actively proliferating. The phenotype was severe even at eclosion and the testes were difficult to dissect. A total of eleven testes could be dissected and all of them contained small Vasa-positive germ cells having the characteristics mentioned above. This result suggested that Rab11-mediated membrane recycling or exocytosis plays a decisive role in the cyst cells in regulating the germ cell divisions. It also raised the possibility that Myosin V/Rab11 could be involved in the exocytosis of a yet-to-be-identified cyst cell-derived signal responsible for regulation of spermatogonial proliferation and differentiation.

DDLC1 is independently required in the cyst cells to maintain cell adhesions. The morphology of cyst cells and their association with the germ cells are vital for cell-cycle arrest in spermatogonia. Recent reports indicate that EGFR signaling in the cyst cell maintains the cell shape by balancing the effects of Rac1 and Rho dependent processes¹⁵. Experimental evidences also suggest that cell adhesion between the cyst cells and the germ cells could play a vital role in the regulation of spermatogonial differentiation^{52,53}. The cell-adhesion and junction-associated proteins, such as Integrins, E-cadherin,

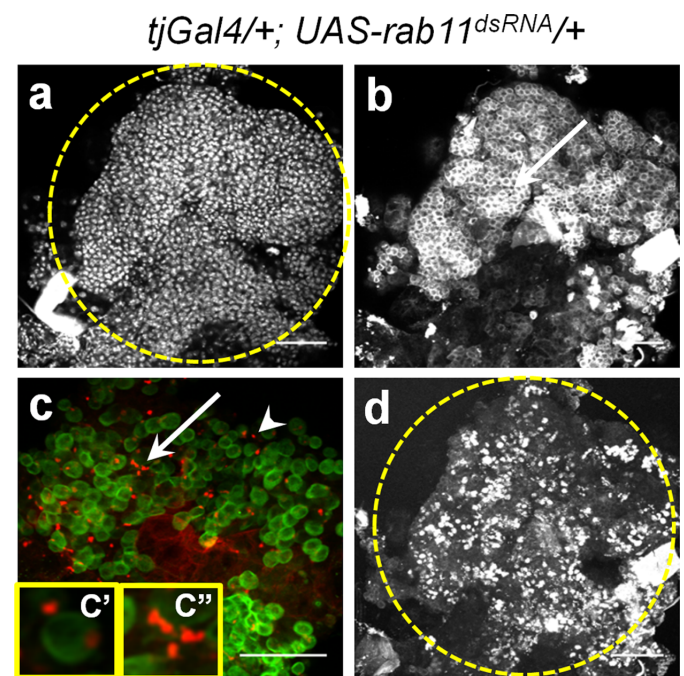


Figure 5 | Disruption of *rab11* in somatic cyst cells using *tjGal4*. Germ cell status was checked in testes from 4 day old *tjGal4/+; UAS-rab11^{dsRNA}/+* flies by anti-Vasa (b, green in c) and anti- α -Spectrin (red in c) staining. Testes were also stained with the Hoescht dye (a) and anti-BrdU (d) after one hour BrdU pulse-labeling. Testes from *tjGal4/+; UAS-rab11^{dsRNA}/+* flies were unusually small and stained brightly with the Hoescht dye (yellow circle, a). In addition, the BrdU label was observed throughout the testis (yellow circle, d) which consisted of only small Vasa-positive cells (arrow, b) bearing spectrosomes (arrowhead in c, inset c') and dumbbell-shaped fusomes (arrow in c, inset c''). Scale bars: 50 μm .



Discs large (DLG1) and β -catenin/Armadillo, are implicated to have a role in cancer progression^{54–56}. DLC1/LC8 is an integral component of both cytoplasmic Dynein and Myosin V, and these proteins are implicated in cellular morphogenesis^{57–59}. Therefore, a partial loss of DDLC1 could also affect cyst cell morphology and cell adhesion.

The cyst cells, marked by the cytosolic GFP localization in *ptcGal4/UAS-eGFP* testis, encapsulated the germ cells (Figure 6A), and this pattern was unchanged in the *ddlc1^{ins1}; ptcGal4/UAS-eGFP* testis (n=10) (Figure 6B). In addition, the DLG1 localization on the spermatogonia and cyst cell interface was unchanged in the *ddlc1^{ins1}* testis (n=7 for *ddlc1^{ins1}*) (Figures 6C, D). However, the Armadillo and DE-cadherin localizations, which mark the cyst and germ cell boundaries in wild-type testis (Figures 6E, G), were abolished in the *ddlc1^{ins1}* testis (Figure 6F, H) (n=9 for Armadillo; n=8 for DE-cadherin). Interestingly, both the Armadillo and DE-cadherin antibodies continued to stain the hub cells in the *ddlc1^{ins1}* testis, indicating that mutations in *ddlc1* selectively affect cell-adhesion in the cysts. In addition, Integrin- β PS, which is present on both spermatogonial and cyst cell membranes (inset, Figure 6I; n=5), was either absent or highly disorganized at the cyst cell perimeters in *ddlc1^{ins1}* testis (Figure 6J; n=19). All these defects were partly restored in the *ddlc1^{ins1}; tjGal4/UAS-mycPIN* testis (Supplementary Figure S4-I), suggesting that DDLC1 is required in the cyst cells for maintaining certain cell adhesion complexes.

The Armadillo and Integrin- β PS localizations were, however, unaffected in both the *tjGal4/UAS-Dhc64C^{dsRNA}* (n=5 for Armadillo; n=6 for Integrin- β PS) and *tjGal4/+; UAS-didum^{dsRNA}/+* testes (n=7 for Armadillo; n=4 for Integrin- β PS), and the DE-cadherin staining was only marginally affected in the *tjGal4/+; UAS-didum^{dsRNA}/+* (n=5) testis (Supplementary Figure S4-II). Since spermatogonial hyperproliferation also occurs in these genotypes, these observations ruled out a causative role of these proteins in the control of spermatogonial proliferation. This was also confirmed by the cyst cell-specific knockdown of Armadillo, DE-cadherin and Integrin- β PS, which failed to cause spermatogonial hyperproliferation (Supplementary Figure S4-III). In many ways, these results clarified a previous evidence¹⁵, which had suggested that gross disorganization of cyst cell enclosures would deregulate spermatogonial divisions. It also showed that the generation of somatic signal in the cyst cells and regulation of germ cell proliferation may work independently of cell adhesion and cell shape determination events.

Discussion

Transition of actively dividing stem cell progeny to the differentiated spermatocytes is one of the key steps in spermatogenesis. The results obtained in this study suggest that the 8kDa conserved light chain DDLC1/LC8 regulates this transition by restricting the spermatogonial divisions through the modulation of somatic microenvironment. DDLC1-dependent functions of Myosin V and cytoplasmic Dynein in the cyst cells induce Bam expression in spermatogonia. In addition, DDLC1 is involved in maintaining certain cell adhesion complexes between the cyst cells and the spermatogonia. DDLC1/LC8 is an essential part of the functional Dynein motor⁶⁰, and the latter is implicated in endosomal transport of EGFR signaling components^{19,61}. Furthermore, the abnormal spermatogonial proliferation caused due to cyst cell-specific removal of DDLC1, Dhc64C or Myosin V is similar to that observed earlier due to the perturbation of EGFR functions in the cyst cells¹⁴. The EGFR and D-raf mediated pathways in the cyst cells also arrest spermatogonial proliferation through an induction of Bam expression in spermatogonia^{6,13,14}. There are two known downstream effectors of *Drosophila* EGFR pathway, the Downstream receptor kinase (Drk)⁶² and Vav⁶³. The latter activates Rac1 in the cyst cells to regulate spermatogonial proliferation and induce spermatocyte differentiation¹⁵. Therefore, the loss of DDLC1 in the cyst cells could inactivate Dynein-based

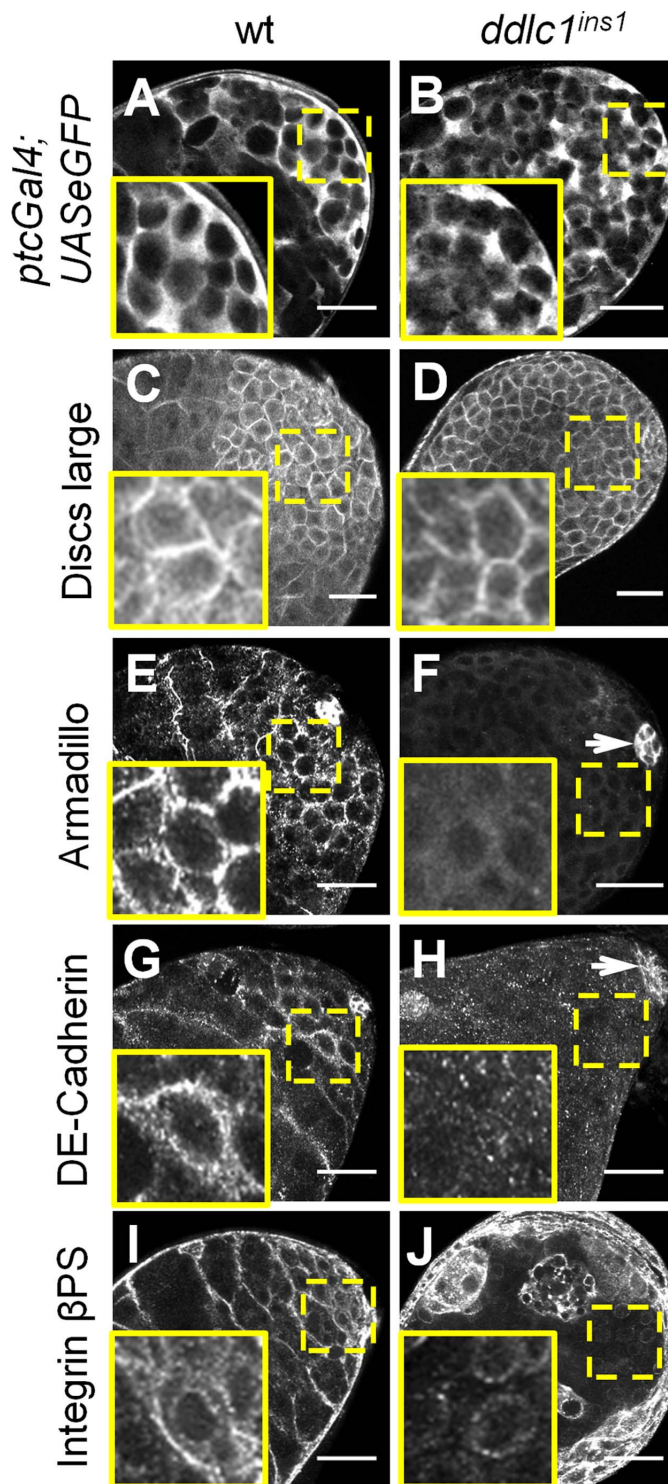


Figure 6 | Status of cell adhesions in wild type and *ddlc1^{ins1}* testes. Testes from 4 days old wild type (A, C, E, G, I) and *ddlc1^{ins1}* (B, D, F, H, J) flies were stained with antibodies against Discs large (C, D), Armadillo (E, F), DE-cadherin (G, H) and Integrin- β PS (I, J). Cyst cell cytoplasm was also visualized by *ptcGal4, UASeGFP* expression in (A) wild type and (B) *ddlc1^{ins1}* testes. Insets show magnified regions of the testes marked with the yellow dashed squares. Scale bars: 25 μ m.

endosomal transport and affect the downstream activation of EGFR signaling. Further investigations are required to test this hypothesis.

DDLC1 is an integral component of Myosin V, which is involved in the F-actin based polarized transport of secretory vesicles and membrane recycling components^{27–29,64,65}. We showed that cyst



cell-specific knockdown of *didum* (Myosin V) caused a high level of abnormal spermatogonial proliferation, and *didum* loss-of-function mutation also dominantly enhanced the defect in *ddlc1* mutant backgrounds. These findings suggest that Myosin V-based polarized secretion/exocytosis of the signaling components and membrane recycling inside the cyst cells could restrict the spermatogonial proliferation. The observation that the cyst cell-specific knockdown of Rab11, a partner of Myosin V in apical secretion²⁹, produced an even more severe germ cell proliferation defect than that of DDLC1 and Myosin V loss, further supports this hypothesis. Therefore, we propose that a feedback signal from the cyst cell could be exocytosed to regulate the germ cell divisions. A search for ligands that associate with Myosin V and Rab11 in the somatic cyst cells could uncover the molecular nature of this putative cyst cell-derived signal.

We also discovered that the loss of DDLC1 in the cyst cells affects their differentiation. It represses *Eya* expression and disrupts cell adhesions by DE-cadherin, Armadillo, and Integrin- β PS between the cyst cells and the germ cells. Although, these defects are not linked to the proliferation of spermatogonia, the above data suggests that they play a vital role in spermatocyte differentiation. Cyst cell-specific knock-down of Armadillo alters the cyst morphology, and that of DE-cadherin blocks the formation of large Vasa-positive spermatocyte nuclei in the testis. Interestingly, loss of cytoplasmic Dynein and Myosin V in cyst cells did not affect the cell adhesion complexes as seen in the *ddlc1* mutants. These results highlight a possible motor-independent role of DDLC1 in maintaining physical contacts between the germ cells and their microenvironment. This is not surprising considering that DLC1 has many interacting partners besides Dynein and Myosin V^{22,43,44,46,47}. Previously, a loss of DLC1 function was only thought to reflect the loss of Dynein function. However, an alternative hypothesis has emerged in recent years, which views DLC1 as a dimerization promoter in different protein complexes that could help in assembling large macromolecular complexes⁴¹.

Methods

Drosophila stocks: Canton-S stock was used as wild type control. Stocks were obtained from Bloomington stock centre, Indiana (USA), VDRC (Austria), DGRC (Kyoto) and as generous gifts from the authors listed in Supplementary Table S1. All fly stocks were grown on standard cornmeal agar and sucrose medium at 25°C. Previous study in our laboratory suggested that *ddlc1* mutant phenotypes are temperature sensitive²⁶. Unless otherwise stated, the newly eclosed adult flies were shifted to 29°C for four days before proceeding for immunostaining. This was found to increase the penetrance and expressivity of the mutant phenotypes. Therefore, this protocol was used for all BrdU pulse-labeling experiments in this study. Similar phenotypes were observed in the adults grown at 25°C at 5-days post eclosion.

Immunofluorescence studies: The testes were dissected in phosphate-buffered saline (PBS)²⁶ and then fixed for one hour in 4% paraformaldehyde (PFA) made in PBS. The samples were then washed four times (15 minutes per wash) with 0.3% PBTX (PBS containing 0.3% Triton X-100) before overnight incubation (12–14 hours) in primary antibody at 4°C. The samples were then washed three times (15 minutes per wash) with 0.3% PBTX and incubated with appropriate secondary antibody solutions for two hours at room temperature. After another three washes in 0.3% PBTX, the samples were mounted on slides with a drop of 70% glycerol and stored at 4°C until imaging. Nuclei were stained by incubating the immunostained tissue in either 1 μ g/ml DAPI (Sigma Chemicals Co., St. Louis, MO) or Hoechst (Invitrogen) for 15 minutes just prior to mounting the samples on slide. Bam immunostaining was performed by following established procedures¹¹. A description of primary antibodies used in this study is presented in Supplementary Table S2.

DDLC1 immunostaining protocol: Dissected testes were fixed in chilled methanol (kept at -20°C) for 10 minutes followed by incubation in 4% formaldehyde at room temperature for 20 minutes. Rest of the immunostaining procedure was identical to the one described above for other immunofluorescence studies.

BrdU pulse labeling and immunofluorescence: We developed a BrdU immunostaining protocol using alkaline denaturation to increase chromatin accessibility of the anti-BrdU antibody. For BrdU pulse, the testes samples were incubated in 10 μ M BrdU solution in PBS for one hour, rinsed once in PBS, and then fixed for one hour in 4% PFA containing 0.2N NaOH. To prepare the fixative, 8% paraformaldehyde (PFA, Sigma Chemicals Co., MO, USA) was dissolved in PBS at 60°C, and then mixed with equal volume of 0.4N NaOH. Rest of the immunostaining

procedure was identical to the one described above for other immunofluorescence studies and involved no extra steps.

Statistical methods: Statistical significance of the differences in average S-phase indices between testes of various genotypes was estimated using the Mann Whitney U test.

Imaging: Fluorescence images were obtained using Olympus Fluoview FV1000 confocal microscope. Optical sections were obtained at 1 μ m intervals. The resulting images were processed using FV10-ASW viewer (version 2.1), Image J® (<http://rsbweb.nih.gov/ij>) and Adobe Photoshop 7.0®.

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Author contributions

KR and PJ planned and executed the project, AGR made the first set of observations, PJ confirmed and further extended it through extensive experiments, PJ, AGR and KR wrote the manuscript together.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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