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The male stem cell niche of *Drosophila melanogaster*: Interactions between the germline stem cells and the hub

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ABSTRACT

The *Drosophila* male stem cell niche is a well characterized structure in which a small cluster of somatic cells send self-renewal signals to neighbouring germ cells. Although the molecular information involved in the stem cell fate have been identified, much less is understand on the mechanisms driving their short-range specific release. Our ultrastructural analysis reveals distinct protrusions of the stem cell plasma membrane that interdigitate with membrane protrusions of the facing hub cells. Some of these protrusions are very elongated and extend into the hub and could correspond to the Mt-Nanotubes. Therefore, the interface between the stem cells and the hub appears more complex than previously reported and the membrane protrusions of the stem cells might represent specialized surface areas involved in the niche-stem cell communication. We also noticed the presence of clathrin-coated vesicles in the germline plasma membrane that might be also involved in delivering information from the hub.

1. Introduction

One of the best-known stem cell niche systems is restricted to the apical tip of the *Drosophila* testis. Here, a crown of 8–10 germline stem cells (GSCs) surrounds a cluster of small post-mitotic somatic cells, the hub [1]. The GSCs divide asymmetrically to produce two daughters, one daughter remains attached to the hub and maintains stem cell fate, while the other daughter, the gonialblast, is displaced away and initiates the differentiation program. A pair of somatic cyst stem cells (CySCs) surrounds each GSC and divide asymmetrically to produce a pair of somatic stem cell daughters and two cyst cells that surround the gonialblast. The different fate of the stem cell progeny is ensured by upstream signals that travel within the niche microenvironment triggering stemness or differentiation. In *Drosophila*, the hub represents the source of two main self-renewal factors, the cytokine-like ligand Unpaired (Upd) and the Bone Morphogenetic Protein ligand Decapentaplegic (Dpp). Upd through its receptor Domeless (Dome) activates the Janus Kinase Transducer and Activator of Transcription (JAK/STAT) [2] pathway that supports GSC maintenance by promoting the stem cell adhesion to the hub [3]. The Dpp signal that is supplied by both the hub and the CySCs [4] activates the R-Smad transcription factor Mother against Dpp (Mad) that promotes GSC fate by preventing the expression of the specific differentiation gene *Bam-of-marbles (Bam)* so that *Bam* is silenced in GSCs but active in gonialblasts [5]. Ectopic expression of *Bam* in GSCs induce, indeed, stem cell loss through premature differentiation [6].

Because the self-renewal signals would behave as morphogens that are usually acting over large distances, it is unclear how these diffusible molecules are restricted to stem cells, and do not reach their differentiating daughters in the proximity of the niche. Extracellular matrix components could maintain active the self-renewal signals, such as Upd, within a cell-diameter and make them ineffective farther [7,8]. It has been also suggested that BMP niche signals are transduced at discrete sites associated with adherens junctions at the interface between the hub and the GSCs [9]. Thus, the adhesion of the GSCs to the hub is essential to ensure the close proximity need to warrant the proper reception of the diffusible signal molecules released by the hub. The adherens junctions also provide a polarity cue for the orientation of the GSC spindle during the asymmetric division. This stereotypical orientation requires Apc2, the *Drosophila* homolog of the human adenomatous polyposis coli protein that interacts with the aster microtubules and β−catenin, thus providing a bridge between the mother centrosome and the adherens junctions [10,11]. Apc2 is mislocalized upon the expression of dominant-negative E-cadherin and the orientation of the centrosome is perturbed [12]. Moreover, a Bazooka (Par-3) cluster that is required for centrosome docking to the cell membrane forms at the GSC/hub interface in an E-cadherin-dependent manner [13]. Although, these findings suggest that the interface between the hub and the GSCs

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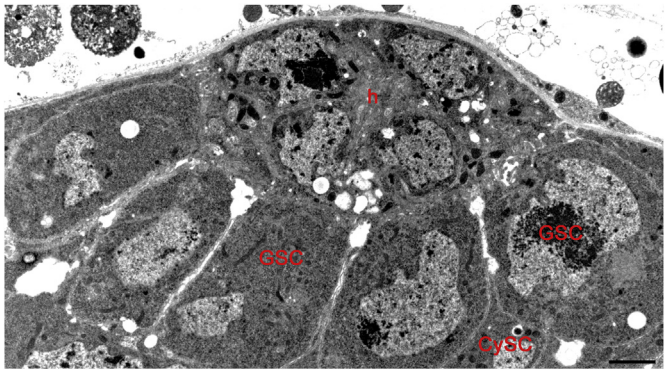

Fig. 1. The *Drosophila* male stem cell niche. h, hub; GSC, germline stem cells; CySC, somatic cyst stem cells. The apical region of the testis is up. Bar: 1 µm.

represents a key region for stem cell maintenance, the morphology of this region is poorly understood. The few ultrastructural studies of the apical region of the testis were made by low magnification electron microscopy (EM) analysis [1,14–16] and do not supply detailed information on the contact area between the hub and the GSCs. However, a careful *in vivo* analysis of the niche has recently revealed the presence of elongated Mt-dependent projections, called Mt-Nanotubes, which emerge from the stem cells and extend into the hub [17]. Mt-Nanotubes might represent specialized cell surface protrusions that mediate the Dpp signalling.

We show here that the plasma membrane of the GSCs displays an intense endocytosis activity and form distinct protrusions that interdigitate with the plasma membrane of the hub cells or extended within the hub region. Therefore, these findings open new routes on the interpretation of how the self-renewal signals produced by the hub can selectively reach the GSCs and no other cells in the proximity of the niche and point to the involvement of distinct surface protrusions in the delivery of specific signals.

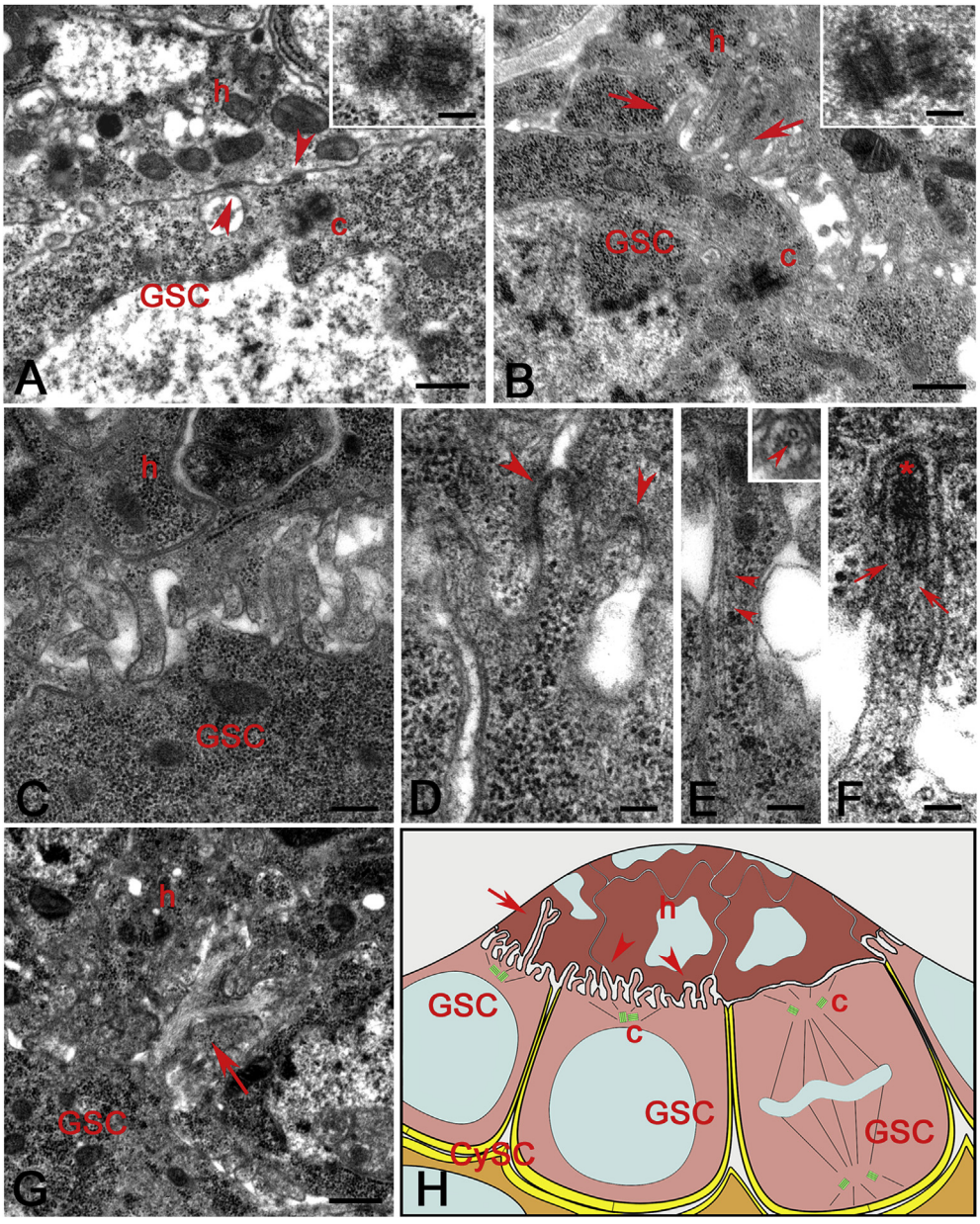

Fig. 2. Membrane protrusions at the interface between the germ stem cells and the hub. The apical surface of the GSCs in contact with the hub is flattened with short adherens junctions (A, arrowheads) or show distinct protrusions (B, arrows); details of centriole pairs in the apical region of the GSCs (A,B, insets). (C) Protrusions at the interface between the GSCs and the hub. (D) The surface protrusions have variable morphologies and their distal tips often contain a dense material (arrowheads). Details of protrusions containing microtubules (E, arrowheads, inset) and microfilaments (F, arrows) that end in a dense material at the tip of the protrusion (asterisk). (G) Some GSCs have long and branched protrusions (arrows) that project into the hub. (H) Schematic representation of the male stem cell niche: h (brown), hub cells; GSC (pink), germline stem cells; CySC (yellow), somatic stem cells; short (arrowheads) and long (arrow) protrusions; c, centrioles. The apical region of the testis is up in all panels. Bars: A,B,G, 0.4 µm; C, 0.2 µm; D-F, 0.1 µm; H, 0.3 µm.

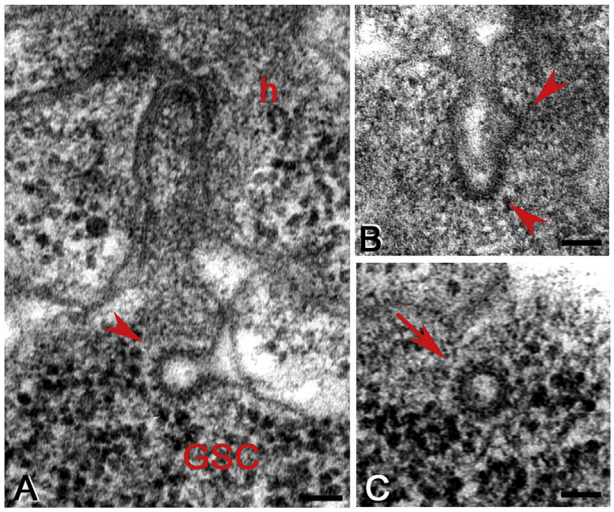

Fig. 3. Endocytosis in the germ stem cells. Plasma membrane at the interface between GSCs and the hub showing forming vesicles (A,B, arrowheads) and vesicles within the apical cytoplasm (C, arrow): the cytoplasmic face of the invaginating membrane is coated by thin evenly spaced dots that look like elements of the clathrin scaffold. h, hub; GSC, germline stem cells. The apical region of the testis is up in all panels. Bars, 0.1 μm.

2. Materials and methods

2.1. Fly stocks

The *Drosophila melanogaster* Oregon-R stock was maintained on standard agar-cornmeal medium in a 12/12 light/dark cycle at 24 °C.

2.2. Transmission electron microscopy

Testes from pupae and adults were dissected in phosphate buffered saline (PBS), and fixed in 2.5% glutaraldehyde in PBS overnight at 4 °C. Samples were post-fixed in 1% osmium tetroxide in PBS for 1 h at 4 °C. The material was then dehydrated through a graded series of ethanol, infiltrated with a mixture of Epon–Araldite resin and polymerized at 60 °C for 48 h. Ultrathin sections were cut with a Reichert ultramicrotome, collected with formvar-coated copper grids, and stained with uranyl acetate and lead citrate. TEM preparations were observed with a Tecnai G2 Spirit EM (FEI, Eindhoven, The Netherlands) equipped with a Morada CCD camera (Olympus, Tokyo, Japan).

3. Results

The stem cell niche of the *Drosophila* testis is composed of a cluster of post-mitotic somatic cells, the hub, surrounded by two stem cell types, the germline stem cells (GSCs) and the somatic cyst stem cells (CySCs) (Fig. 1). Hub cells are small cells with irregularly shaped nuclei and a convoluted plasma membrane that forms intricate interdigitations among the neighbouring cells. These interdigitations increase the mutual cohesion of the hub cells to which GSCs are anchored. GSCs are large cells that display a stereotypical polarity with their apical region in contact with the hub (Fig. 1).

3.1. The apical surface of the GSCs

The male GSCs from third instar larvae or adult testes either showed a flattened apical surface (Fig. 2A) or had a more or less convoluted plasma membrane (Fig. 2B) that formed thin finger-like protrusions that interdigitated with opposite protrusions emerging from the hub cells (Fig. 2C). The protrusions (Fig. 2D–F) had a variable shape and size (0.3–0.7 μm in length and 0.04–0.06 μm in diameter) and contained isolated microtubules (Fig. 2E) or were filled with microfilament bundles that end in apical dense plaques (Fig. 2F). The membrane protrusions covered the whole apical surface of the GSCs, or were restricted to their apical periphery, thus leaving a more or less extended flattened central area at the interface with the hub. We also occasionally find longer branched protrusions (1.70–1.95 μm in length and 0.22–0.26 μm in diameter) that emerged from the apical surface of the GSCs and extended into the hub region (Fig. 2G). Remarkably, dividing GSCs always had a flattened apical surface (Fig. 2H). We also find that interphase GSCs with flattened apical surface had slightly disoriented parent centrioles (Fig. 2A, inset), whereas the GSCs with distinct surface protrusions displayed orthogonal centrioles (Fig. 2B, inset).

Higher magnification of the apical surface of the GSCs uncovered an intense endocytic membrane trafficking. We find, indeed, invaginating vesicles at the basis of the membrane projections (Fig. 3A) or along the wall of short membrane pockets where several vesicles formed (Fig. 3B). Vesicle formation required clathrin coating (Fig. 3A and B) that also persisted when the vesicles detached from the plasma membrane (Fig. 3C).

3.2. Junctional contacts within the stem cell niche

Each GSC was surrounded by three plasma membrane domains: an apical domain in contact with the hub; lateral and basal domains in contact with the CySCs (Fig. 2H). When the apical membrane domains were devoid of membrane projections, distinct adherens junctions appeared at the interface between the GSCs and the hub cells (Fig. 4A). These junctional contacts were characterized by an unusual organization: the sub membranous dark area that has about the same dimension on adherens junctions of adjacent epithelial cells, was more prominent in the cytoplasmic domain of the GSCs (Fig. 4B and C). Moreover, the microfilaments are mostly anchored to the GSC side of the junctions (Fig. 4C). The intercellular space of the adherens junctions was often crossed by thin bridges (Fig. 4C). Such bridges were also associated to the opposite cell membranes close to the junctions lacking the submembranous dark material (Fig. 4B). Evenly spaced bridges often connected large protrusions of the GSCs to the hub cell membrane (Fig. 4D). These areas lacked dark material and cytoplasmic microfilaments.

Thin bridges linking opposite cell membranes were also found far from the adherens junctions. These discrete contact areas look like septate junctions and were observed among GSCs and hub cells (Fig. 4E), GSCs and CySCs (Fig. 4F), and within the hub region (Fig. 4G).

4. Discussion

It is generally retained that the contact area between the GSCs and the hub cells in the male stem cell niche of *Drosophila* is flattened in contrast to other insect species in which the male GSCs have a highly convoluted apical membrane [18]. This concurring opinion results from conventional immunofluorescence (IF) observations and low magnification EM analysis made on the *Drosophila* stem cell niche. We confirm that some GSCs have, indeed, a flat apical surface, but others within the same stem cell niche display distinct membrane protrusions. Most of the protrusions interdigitate with opposite protrusions from the hub cells, whereas some of them are longer and extended within the hub region. These protrusions increase the surface of the apical membrane and may represent specialized areas where short range self-renewal signals produced by the hub can be transferred to the stem cells.

The long protrusions that extend from GSCs into the hub could correspond to the Mt-Nanotubes found *in vivo* observations of the *Drosophila* stem cell niche [17]. However, we failed to observe microtubule bundles within these protrusions, whereas distinct microtubules were found in the peripheral cytoplasm of the GSCs. This apparent

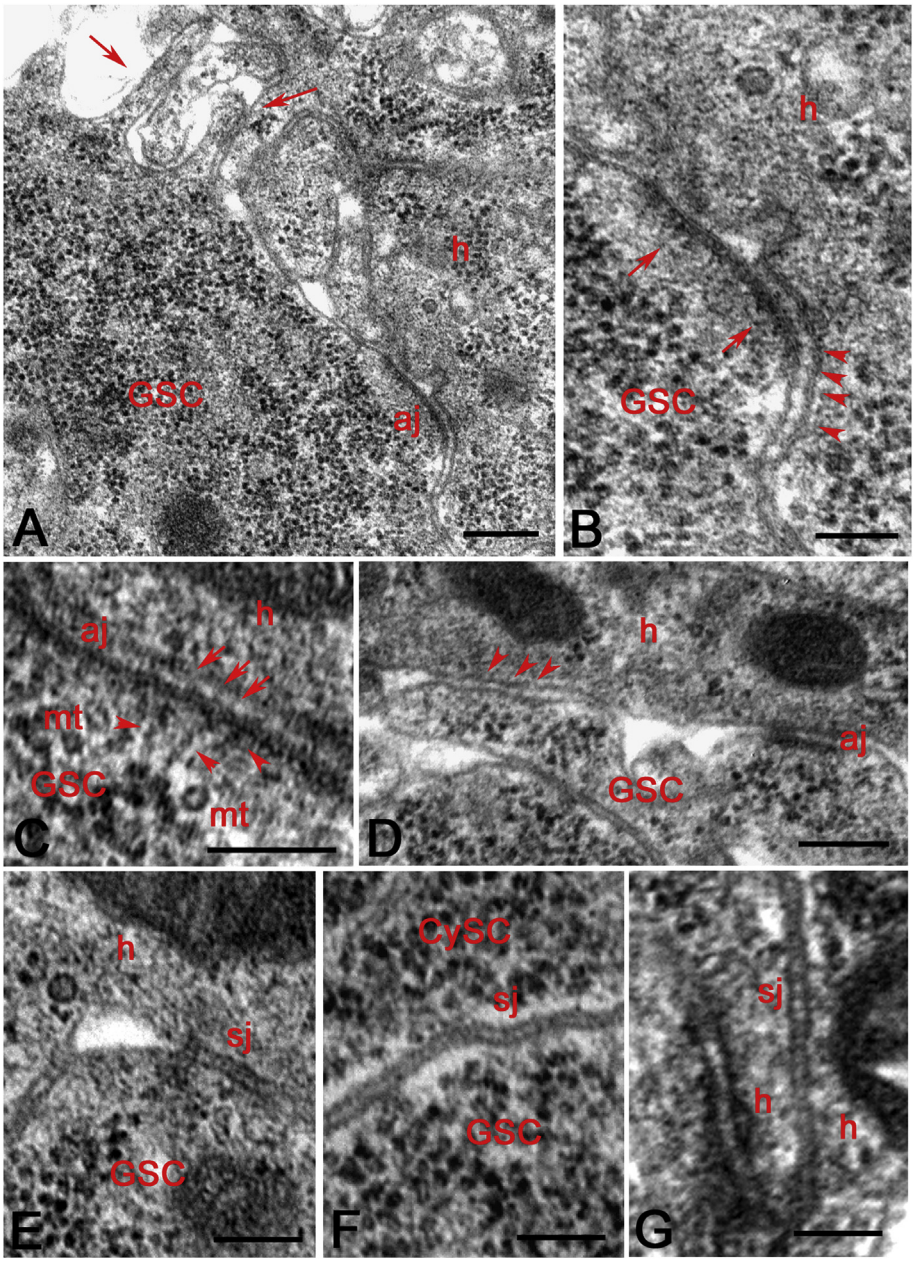

Fig. 4. Junctional contacts in the stem cell niche. (A) Apical region of a GSC showing distinct protrusions (arrows) and isolated adherens junctions (aj). (B) Detail of the adherens junction in (A): a dense material (arrows) mainly accumulates in the cytoplasm side of the GSC and thin bridges (arrowheads) cross the intercellular space between the GSCs and the hub. (C) High magnification of a typical adherens junction (aj) between a GSC and the hub showing the asymmetric distribution of the dense material and the thin bridges linking the opposite cell membranes (arrows); some microfilaments (arrowheads) end to the dense material at the cytoplasmic side of the GSC; mt, microtubules. (D) Detail of facing GSC and hub cells in which the contact is established by thin bridges (arrowheads) and small adherens junctions (aj). Details of septate junctions (sj) between GSCs and hub cells (E); between GSCs and CySCs (F); within the hub cells (G). h, hub; GSC, germline stem cells; CySC, somatic cyst stem cells. The apical region of the testis is up in all panels. Bars: A, 0.2 μm; B-G, 0.1 μm.

discrepancy, may be due to the sensitivity of the Mt-Nanotubes to the conventional fixation procedures or to their fast turnover that makes very difficult their detection in fixed material.

The coated vesicles enriched at the apical membrane of the GSCs point to a clathrin-mediated endocytosis process which may represent the hallmark of early endocytic trafficking events. It has been shown that the endocytic machinery is essential for intercellular signalling during tissue morphogenesis and development [19–22]. Thus, the coated vesicles may represent an additional route for the local release of the signals produced by the hub.

The shorter surface protrusions might be also involved in the assembly of the adherens junctions at the interface between the GSCs and the hub. Filopodial protrusions harbouring at their tips dense plaques for microfilament anchoring have been found between opposing epithelial cells during the process of dorsal closure of the *Drosophila* embryo [23]. These protrusions join together the epithelial cells and later resolve in permanent contact regions [24], thus reflecting different stages of junctional maturation [25]. [26]. The initial steps of the adherens junction assembly in the stem cell niche may be very similar to those described during the tissue morphogenesis of the *Drosophila* embryo [27]. The thin bridges that connected the opposite cells membranes of opposite GSCs and hub cells may reflect orderly clusters of the transmembrane adhesive components cadherins [28]. E-cadherins are, indeed, largely expressed in the *Drosophila* testis at the interface among the GSCs and the hub [12]. This interaction might precede the assembly of the dark sub membranous regions to which the actin filament bundles attach. Unlikely, epithelial cells in which the adherens junctions display a symmetric organization, the dark sub membranous region associated with the cytoplasmic side of the GSCs is larger than that found within the hub cell side of the junction. This suggest that the completion of the adhesion contacts is delayed in the hub cells and do not require concerted mechanisms. Actin filament bundles are, indeed, more prominent within the cytoplasmic side of the GSCs. The adherens junctions found between the GSCs and the hub do not resolve in permanent adhesion junctions as observed in epithelial *Drosophila* cells during the process of dorsal closure but they are highly dynamic structures. We find that the membrane protrusions are enriched at the surface of several interphase cells, but most of them disappear at the

onset of cell division when the contact area between the GSCs and the hub flattens. The number of the adherens junctions increases concurrently to the reduction of the surface protrusions, thus enhancing the adhesion of the GSCs to the hub during cell division.

Some core septate junction components, specifically Discs large (Dlg), Neurexin-IV (Nrx-IV) and Coracle (Cora), are diffusely localized at the interface of the cells that form the apex of the larval testis including the hub, GSCs and SSCs [29,30]. We, indeed, find contact regions in which the opposite membranes were linked by thin bridges morphologically resembling the septate junctions that are usually found in most somatic cells of the arthropod tissues [31]. However, the septate junctions have a distinctive architecture characterized by evenly spaced transversal bridges that link adjacent cell membranes through a nearly constant intercellular space. Conversely, the intercellular space and the distance interval among the bridges observed in the male stem cell niche of *Drosophila* were not constant. Therefore, the septate junctions fail to reach a final organization in the stem cell niche. Moreover, the typical septate junctions were found along the lateral membranes of adjacent cells, whereas in the male stem cell niche they mainly link the apical membranes of opposing cells. Unlikely canonical septate junctions that are mainly involved in ensuring stable cell-to-cell adhesion, the septate junction observed within the *Drosophila* stem cell niche may be only marginally required for this function. GSC maintenance mainly depend, indeed, by the adherens junction associated protein E-cadherin that mediate stem cell anchorage to the niche [10,12]. However, the septate junctions between the somatic cyst cells of the fly testis form an occluding barrier that is essential for spermatogenesis. Removing these contact regions lead, indeed, to the overgrowth of the hub and to spermatogenesis failure [32].

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

M.G.R. and G.C. conceived the project. V.P. and M.G.R. performed experiments. G.C. wrote the manuscript.

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References

[1] R.W. Hardy, K.T. Tokuyasu, D.L. Lindsley, M. Garavito, The germinal proliferation center in the testis of *Drosophila melanogaster*, J. Ultrastruct. Res. 69 (1979) 180–190.
[2] N. Tulina, E. Matunis, Control of stem cell self-renewal in *Drosophila* spermatogenesis by JAK-STAT signalling, Science 294 (2001) 2546–2549.
[3] J.L. Leatherman, S. Dinardo, Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in *Drosophila* testes, Nat. Cell Biol. 12 (2010) 806–811.
[4] E. Kawase, M.D. Wong, B.C. Ding, T. Xie, Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the *Drosophila* testis, Development 131 (2004) 1365–1375.
[5] D. Chen, D. McKearin, Dpp signaling silences *bam* transcription directly to establish asymmetric divisions of germline stem cells, Curr. Biol. 13 (2003) 1786–1791.
[6] X.R. Sheng, C.M. Brawley, E.L. Matunis, Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the *Drosophila* testis, Cell Stem Cell 5 (2009) 191–203.
[7] Y. Hayashi, S. Kobayashi, H. Nakato, *Drosophila* glypicans regulate the germline stem cell niche, J. Cell Biol. 187 (2009) 473–480.
[8] D. Yan, X. Lin, Shaping morphogen gradients by proteoglycans, Cold Spring Harb. Perspect. Biol. 1 (2009) a002493.
[9] M. Michel, I. Raabe, A.P. Kupinski, R. Pérez-Palencia, C. Bökel, Local BMP receptor activation at adherens junctions in the *Drosophila* germline stem cell niche, Nat. Commun. 2 (2011) 415.
[10] Y.M. Yamashita, D.L. Jones, M.T. Fuller, Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome, Science 301 (2003) 1547–1550 2003.
[11] Y.M. Yamashita, A.P. Mahowald, J.R. Perlin, M.T. Fuller, Asymmetric inheritance of mother versus daughter centrosome in stem cell division, Science 315 (2007) 518–521.
[12] M. Inaba, H. Yuan, V. Salzmann, M.T. Fuller, Y.M. Yamashita, E-cadherin is required for centrosome and spindle orientation in *Drosophila* male germline stem cells, PLoS One 5 (2010) e12473.
[13] M. Inaba, Z.G. Venkei, Y.M. Yamashita, The polarity protein Baz forms a platform for the centrosome orientation during asymmetric stem cell division in the *Drosophila* male germline, Elife (2015), https://doi.org/10.7554/eLife.04960.
[14] M.G. Riparbelli, G. Colozza, G. Callaini, Procentriole elongation and recruitment of pericentriolar material are downregulated in cyst cells as they enter quiescence, J. Cell Sci. 122 (2009) 3613–3688.
[15] S. Srinivasan, A.P. Mahowald, M.T. Fuller, The receptor tyrosine phosphatase Lar regulates adhesion between *Drosophila* male germline stem cells and the niche, Development 139 (2012) 1381–1390.
[16] M. Gottardo, G. Callaini, M.G. Riparbelli, The *Drosophila* centriole - conversion of doublets into triplets within the stem cell niche, J. Cell Sci. 128 (2015) 2437–2442.
[17] M. Inaba, M. Buszczak, Y.M. Yamashita, Nanotubes mediate niche-stem-cell signalling in the *Drosophila* testis, Nature 16 (2015) 329–332 523.
[18] D.C. Dorn, A. Dorn, Stem cell autotomy and niche interaction in different systems, World J. Stem Cell. 7 (2015) 922–944.
[19] C. Bökel, M. Brand, Endocytosis and signaling during development, Cold Spring. Harb. Perspect. Biol 6 (2014) a017020 2014.
[20] P.P. Di Fiore, M. von Zastrow, Endocytosis, signaling, and beyond, Cold Spring. Harb. Perspect. Biol 6 (2014) a016865.
[21] S. Eaton, F. Martin-Belmonte, Cargo sorting in the endocytic pathway: a key regulator of cell polarity and tissue dynamics, Cold Spring. Harb. Perspect. Biol. 6 (2014) a016899.
[22] M. Gonzalez-Gaitan, F. Jülicher, The role of endocytosis during morphogenetic signaling, Cold Spring. Harb. Perspect. Biol 6 (2014) a016881.
[23] P. Hayes, J. Solon, *Drosophila* dorsal closure: an orchestra of forces to zip shut the embryo, Mech. Dev. 144 (2017) j.mod.2016.12.
[24] A. Jacinto, S. Woolner, P. Martin, Dynamic analysis of dorsal closure in *Drosophila*: from genetics to cell biology, Dev. Cell 3 (2002) 9–19.
[25] U. Tepass, V. Hartenstein, The development of cellular junctions in the *Drosophila* embryo, Dev. Biol. 161 (1994) 563–596.
[26] V. Vasioukhin, C. Bauer, M. Yin, E. Fuchs, Directed actin polymerization is the driving force for epithelial cell-cell adhesion, Cell 100 (2000) 209–219.
[27] T.J. Harris, Adherens junction assembly and function in the *Drosophila* embryo, Int. Rev. Cell Mol. Biol. 293 (2012) 45–83.
[28] A.S. Yap, G.A. Gomez, R.G. Parton, Adherens junctions revisualized: organizing cadherins as nanoassemblies, Dev. Cell 35 (2015) 12–20.
[29] F. Papagiannouli, B.M. Mechler, Discs large regulates somatic cyst cell survival and expansion in *Drosophila* testis, Cell Res. 19 (2009) 1139–1149.
[30] M.J. Fairchild, C.M. Smendziuk, G. Tanentzapf, A somatic permeability barrier around the germline is essential for *Drosophila* spermatogenesis, Development 142 (2015) 268–281 2015.
[31] S. Banerjee, A.D. Sousa, M.A. Bhat, Organization and function of septate junctions: an evolutionary perspective, Cell Biochem. Biophys. 46 (2006) 65–77.
[32] M.J. Fairchild, L. Yang, K. Goodwin, G. Tanentzapf, Occluding junctions maintain stem cell niche homeostasis in the fly testes, Curr. Biol. 26 (2016) 2492–2499 2016.