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Multipotent somatic stem cells contribute to the stem cell niche in the *Drosophila* testis

Justin Voog^{1,2}, Cecilia D'Alterio¹, and D. Leanne Jones¹

¹Laboratory of Genetics, The Salk Institute for Biological Studies, San Diego, La Jolla, California 92037, USA

²Department of Biomedical Sciences, University of California, San Diego, La Jolla, California 92037, USA

Abstract

Adult stem cells reside in specialized microenvironments, or niches, that have an important role in regulating stem cell behaviour¹. Therefore, tight control of niche number, size and function is necessary to ensure the proper balance between stem cells and progenitor cells available for tissue homeostasis and wound repair. The stem cell niche in the *Drosophila* male gonad is located at the tip of the testis where germline and somatic stem cells surround the apical hub, a cluster of approximately 10–15 somatic cells that is required for stem cell self-renewal and maintenance^{2–4}. Here we show that somatic stem cells in the *Drosophila* testis contribute to both the apical hub and the somatic cyst cell lineage. The *Drosophila* orthologue of epithelial cadherin (DE-cadherin) is required for somatic stem cell maintenance and, consequently, the apical hub. Furthermore, our data indicate that the transcriptional repressor *escargot* regulates the ability of somatic cells to assume and/or maintain hub cell identity. These data highlight the dynamic relationship between stem cells and the niche and provide insight into genetic programmes that regulate niche size and function to support normal tissue homeostasis and organ regeneration throughout life.

Many stem cell niches include support cells that influence stem cell behaviour through secretion of diffusible molecules. Physical contact between stem cells and support cells and/or the extracellular matrix holds stem cells within the niche and close to self-renewal signals. Furthermore, niches provide spatial and mechanical cues that influence the fate of stem cell daughters. Therefore, the stem cell niche has an important role in regulating stem cell maintenance, self-renewal and survival (reviewed in ref. 5). However, little is known about the factors that regulate niche maintenance or size.

Approximately ten somatic cells, called the hub, are found at the apical tip of the *Drosophila* testis (Fig. 1a)². Germline stem cells (GSCs) and somatic stem cells (SSCs) surround and are in contact with hub cells. Whereas GSCs sustain spermatogenesis, SSCs produce cyst cells that encapsulate the maturing germ cells and ensure differentiation^{6,7}. Hub cells secrete the growth factor Unpaired (Upd)^{3,4}, which activates the JAK-STAT signal transduction pathway in adjacent stem cells. JAK-STAT signalling is necessary for stem cell maintenance and is sufficient to specify self-renewal of both GSCs and SSCs in the testis^{3,4,8}.

The apical hub is typically described as a post-mitotic, static structure. However, in agametic flies, SSCs proliferate and express hub markers, leading to an apparent expansion of the apical

Correspondence and requests for materials should be addressed to D.L.J. (ljones@salk.edu).

Author Contributions

J.V. and D.L.J. planned experiments; J.V. and C.D'A. performed experiments and analysed data; D.L.J. wrote the manuscript; and J.V. and C.D'A. edited the manuscript.

hub9. Furthermore, recent studies have noted that hub cell number and function decrease with age^{10,11}, indicating that the stem cell niche in the testis is dynamic. Hub cells and SSCs share numerous features, including similar gene expression patterns and close association with GSCs and each other^{2,12}; however, the precise relationship between SSCs and hub cells has not been explored.

We proposed that SSCs may serve as a source of cells that contribute to the apical hub and, consequently, the stem cell niche. To address whether SSCs give rise to hub cells, positively marked β -galactosidase-expressing (β -gal⁺) SSCs were generated using mitotic recombination, a technique typically used for lineage tracing analyses. Labelled SSCs were generated by heat-shocking flies of the appropriate genotype to initiate FLP-mediated recombination, resulting in reconstitution of the *α -tubulin* promoter upstream of the *lacZ* gene¹³. Hub cells were identified by immunolabelling with antibodies to Fasciclin III (FasIII, Fig. 1b, third panel)¹⁴ or DE-cadherin (Fig. 1c)¹⁵, cell-surface proteins concentrated at hub cell junctions. SSCs and early cyst cells were identified by immunolabelling with antibodies to Traffic Jam (TJ), a transcription factor that is strongly expressed in early cyst cell nuclei¹⁶ (Fig. 1b, f, first panels) and weakly expressed in hub cells (Fig. 1f, fourth panel).

Wild-type SSC clones were identified as β -gal⁺ cells adjacent to the apical hub and surrounding germ cells (Fig. 1c and Supplementary Table 1). A series of heat shocks after eclosion (hatching) led to at least one β -gal⁺ SSC in 57% ($n = 47$) of testes from 3-day-old males analysed 1 day after heat shock. At 5 days after heat shock, 28% ($n = 74$) of testes contained at least one β -gal⁺ SSC, and this frequency decreased to 10% ($n = 183$), 10% ($n = 225$) and 4% ($n = 142$) at 10, 15 and 30 days after heat shock, respectively (Supplementary Table 1).

In addition to β -gal⁺ SSCs, β -gal⁺ hub cells were also observed that co-labelled with DE-cadherin and FasIII. In fact, β -gal⁺ cells were found within the hub in 34% ($n = 47$), 47% ($n = 74$) and 60% ($n = 225$) of testes from males at 1, 5 and 15 days after heat shock, respectively (Fig. 1e, f and Supplementary Table 2). No β -gal⁺ cells were observed in flies not exposed to the heat-shock protocol (Supplementary Table 2).

Previous reports concluded that hub cells are post-mitotic^{2,9}; however, it is possible that hub cells undergo rare divisions to become marked during recombination. To test whether hub cells are mitotic, dividing cells in the testis were labelled with 5'-bromo-2-deoxyuridine (BrdU), which is incorporated into newly synthesized DNA during S phase. Flies were fed ('pulsed') BrdU for 30 min and aged ('chased') for up to 15 days. Subsequently, labelled testes were costained with antibodies to BrdU, as well as to the hub marker FasIII. No BrdU-positive (BrdU⁺) hub cells were detected after a 1-day chase ($n = 82$), although cells adjacent to the hub were clearly labelled (Fig. 2a).

However, BrdU⁺ hub cells were observed 3-10 days after labelling (Fig. 2b, d). BrdU⁺ hub cells were present in 4% ($n = 143$), 8% ($n = 103$) and 3% ($n = 96$) of testes assayed at 5, 7-8 and 10 days after labelling (Supplementary Table 3). Moreover, these BrdU⁺ cells co-expressed FasIII (Fig. 2b) and an *upd* reporter, indicating that these cells function as hub cells (Fig. 2d). These data are consistent with previous results indicating that hub cells are post-mitotic^{2,9} and support the hypothesis that mitotically active SSCs act as a source of cells that can contribute to the apical hub.

SSCs are reported to be the only dividing somatic cells in the testis; however, we observed two distinct populations of somatic cells dividing near the testis tip. One group, which constituted 69% (20 out of 29) of phospho-histone-H3-positive somatic cells ($n = 453$ testes), appeared to be immediately adjacent to the hub, similar to GSCs (Fig. 3a, b). We also observed somatic cells that were dividing 1-2 cell distances away from the hub (9 out of 29) (Fig. 3c, d). Several scenarios could explain these observations: there are two SSC populations, one which gives

rise to hub cells and another that sustains the cyst cell lineage, or there are SSCs that produce both hub cells and a transient amplifying somatic cell population.

To distinguish between these possibilities, we adjusted the heat-shock regime to label, on average, only one β -gal⁺ somatic cell and analysed the clones derived from these marked cells (Supplementary Fig. 1). Thirteen per cent of testes examined contained marked somatic cells adjacent to the hub at 1 day after heat shock ($n = 185$), which decreased to 9.5% ($n = 190$), 3.3% ($n = 212$) and 2.8% ($n = 72$) of testes at 5, 10 and 15 days after heat shock, indicating a half-life for SSCs between 5-10 days. Single, marked somatic cells displaced away from the hub were found initially in 11.3% of testes examined at 1 day after heat shock, but this number decreased to 0.5% by 5 days after heat shock, which is consistent with these cells being transient non-stem-cell clones (Fig. 3e, f and Supplementary Fig. 1). In contrast, the number of testes that contained marked hub cells increased from 11.9% at 1 day after heat shock to 25.8%, 24.5% and 18.1% at 5, 10 and 15 days after heat shock, respectively. Clones containing all three cell types were observed in 22% ($n = 18$) and 14% ($n = 7$) of testes that contained marked SSCs at 5 and 10 days after heat shock, respectively (Fig. 3g-i). From these data we conclude that multipotent SSCs self-renew and contribute to both hub and cyst cell lineages, whereas dividing cyst cells, which we call cyst progenitor cells (CPCs), expand the pool of cells capable of encapsulating newly divided gonialblasts and maturing spermatogonia to ensure terminal differentiation^{6,7,17} (Supplementary Fig. 1).

To identify factors required for incorporation of cells into the apical hub, SSCs were generated that were mutant for genes expressed in both cell types: DE-cadherin, which is encoded by the *shotgun* (*shg*) gene, and the transcriptional repressor Escargot (*Esg*). DE-cadherin is expressed in cyst cells and is strongly enriched in the hub (Fig. 1c, d). SSC clones were generated that were homozygous mutant for either the loss-of-function *shg*^{IG29} or amorphic *shg*^{IH} allele. SSC maintenance and frequency of marked hub cells were assayed at various time points. In this experiment, marked cells and their progeny subsequently become permanently labelled by ubiquitous green fluorescent protein (GFP) expression (Fig. 4).

Heat-shocked wild-type testes possessed GFP⁺ GSCs and SSCs, as well as GFP⁺ hub cells that co-stained with FasIII and DE-cadherin (Fig. 4 a-c, e). In contrast to wild type, *shg* mutant GSC and SSC clones were not maintained (Fig. 4d, Supplementary Fig. 2 and Supplementary Table 1), indicating that DE-cadherin has a role in stem cell maintenance in the testis, similar to its role in the ovary^{18,19}, presumably by holding stem cells within the niche and close to self-renewal signals.

Marked hub cells were observed in 14% ($n = 144$), 35% ($n = 114$) and 65% ($n = 110$) of wild-type testes examined at 5, 10 and 15 days after heat shock, respectively (Fig. 4e and Supplementary Table 2). Notably, progeny of DE-cadherin mutant SSCs contributed to the apical hub at a frequency similar to progeny from wild-type SSCs (Fig. 4f and Supplementary Table 2). These data indicate that although DE-cadherin is required for SSC maintenance, it is not absolutely required for mediating the contribution of SSC progeny to the hub.

To confirm that *shg* is not required in hub cells for maintaining the apical hub, we used RNAi-mediated knockdown of *shg* expression in hub cells. A FasIII⁺ apical hub was detected in 100% of testes from 1-day-old ($n = 12$), 10-day-old ($n = 15$) and 20-day-old males ($n = 28$), despite a reduction in DE-cadherin expression in hub cells (Supplementary Fig. 3). Testes collected at 20 days also displayed normal expression of a *upd* reporter (98%, $n = 115$) and contained TJ⁺ (100%, $n = 25$) cells near the apical tip (Supplementary Fig. 3). These data support our findings that DE-cadherin is not absolutely required in hub cells to maintain a functional stem cell niche.

However, *shg* is required in SSCs and early cyst cells for maintaining the apical hub. Knockdown of *shg* in all SSCs and early cyst cells resulted in a decrease in the number of TJ⁺ cells in 1-day-old males, consistent with a role for *shg* in SSC maintenance (Fig. 4g, h and Supplementary Fig. 4). Surprisingly, decreased levels of DE-cadherin were also observed in hub cells (Supplementary Fig. 4). In 29% (*n* = 42) of 15-day-old and 44% (*n* = 36) of 20-day-old males, the apical hub was severely diminished or lost, as determined by FasIII expression (Fig. 4i, j and Supplementary Fig. 4). These data support our model that SSCs act as a source of cells to maintain the apical hub.

The transcriptional repressor Escargot is expressed in many tissues, including GSCs, early cyst cells and hub cells in the testis^{20,21}. Males carrying a viable, hypomorphic allele of *esg*, called *shutoff*, exhibit loss of apical hub cells during development (J.V., manuscript in preparation). Therefore, we hypothesized that *esg* may be required for regulating the contribution of SSCs to the hub. Mutant labelled SSCs were generated using two amorphic *esg* alleles, as described above.

Unlike progeny from *shg* mutant SSCs, progeny from *esg* mutant SSCs did not contribute to the hub at the same frequency as wild-type controls: *esg*^{L2} mutant GFP⁺ hub cells were observed in 5% (*n* = 38), 3% (*n* = 40), 0% (*n* = 36) and 4% (*n* = 52) of testes examined at 1, 5, 10 and 15 days after heat shock, respectively (Supplementary Table 2). In instances when *esg* mutant GFP⁺ hub cells were observed, normal hub morphology was often severely disrupted (Fig. 4k, l). These data suggest that *esg* regulates either the contribution of SSC progeny to the hub, perhaps by facilitating the cell fate transition between SSC and hub cell, or maintenance of hub cell fate.

To explore Esg function further, we used the agametic *oskar* (*osk*) mutant phenotype, in which SSCs proliferate and express hub markers, resulting in an apparent expansion of the apical hub⁹. If Esg is required for mediating the transition of somatic cyst cells to the apical hub, we predicted that the expansion of FasIII⁺ cells would be blocked in an *esg;osk* double mutant background. In contrast to the expansion of FasIII⁺ cells in 82% (*n* = 16) of *osk* mutant testes, only 22% (*n* = 63) of testes from *esg*^{shof};*osk* mutant males showed expansion of FasIII, despite there being clearly more TJ⁺ somatic cyst cells (Fig. 4m, n). These data support our previous results and indicate that *esg* is required for the ability of somatic cells to assume and/or maintain hub cell fate.

Our findings demonstrating that SSCs can adopt a hub cell fate highlight the dynamic nature of the stem cell-niche relationship and provide a mechanism to regulate the size and function of the stem cell niche in the *Drosophila* testis. In our model, as somatic cells are displaced from the hub, there is a decline in self-renewal and proliferation potential, which could be reinforced by encapsulation of differentiating germ cells (Supplementary Fig. 1). Interestingly, expansion of the somatic cyst cells as a consequence of germline loss suggests that germ cells exert an anti-proliferative influence that must be overcome in SSCs.

A better understanding of how stem cell niches are established and regulated in mammalian systems could facilitate modulation of the niche to enhance transplantation of stem cells in regenerative medicine^{22,23}. Conversely, if an expanded or modified niche accompanies tumour progression or metastasis, then blocking niche maintenance programmes (niche ablation) could be used as an important anti-cancer therapeutic^{24,25}.

METHODS SUMMARY

Fly husbandry and stocks

Flies were raised at 25 °C on standard cornmeal-molasses media. The *esg^{shof}* allele was isolated in the Fuller laboratory (J.V. *et al.*, manuscript in preparation). The UAS-*shgRNAi* line was obtained from the Vienna *Drosophila* Resource Center (VDRC). The *updGAL4-UAS-gfp* line was a gift from E. Bach. For additional information see Methods.

Lineage tracing analyses

Adult flies or embryos (*y,w, hs-FLP/+; X-15-29/X-15-33*) were heat shocked for 2 h on 2 consecutive days (maximal clone induction) or for 1 h on 1 day (moderate clone induction) 26.

For MARCM experiments, late pupae or early larvae were heat shocked at 37 °C for 2 h on 2 consecutive days. Flies were collected at various time points after the last heat shock, immunostained and analysed 27.

BrdU labelling

OregonR or *updGAL4-UAS-gfp* 1-day-old males were starved in wet-plugged vials overnight (~16 h). Subsequently, starved flies were moved to vials containing 100 µl of 100 mM BrdU dissolved in grape juice for 30 min and then moved to agar-molasses vials and aged. Testes were stained as described previously 11.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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APPENDIX

METHODS

Fly stocks

The following stocks were used; more information can be found at Flybase (<http://flybase.bio.indiana.edu>): *hs-FLP-12*, *X-15-29*, *X-15-33*, *OregonR*, *hs-FLP-122*, *α-tubulinGAL80*, *α-tubulinGAL4*, *2x-UAS-eGFP*, *FRT40A*, *FRTG13*, *osk^{CE4}*, *osk³⁰¹*, *shg^{IG29}*, *shg^{IH}*, *esg^{G66}* and *esg^{L2}*. Flies of the genotype *esg^{shof}; osk* were generated by crossing *esg^{shof}/CyO*; *osk^{CE4}/osk³⁰¹* females to *esg^{shof}/CyO*; *osk^{CE4}/TM6b*, *Hu* males. Controls were progeny from *esg^{shof}/CyO*; *osk^{CE4}/TM6b*, *Hu* or *esg^{shof}/CyO*; *osk³⁰¹/TM6b*, *Hu* females crossed to *esg^{shof}/CyO*; *osk^{CE4}/TM6b*, *Hu* males. The UAS-*shgRNAi* stock was obtained from the Vienna *Drosophila* RNAi Center.

MARCM analysis

MARCM wild-type control genotypes used were: *y,w, hsflp¹²²; FRT40A/FRT40A tubGAL80*; *tubGAL4/2x-UAS-eGFP* and *y,w, hsflp¹²²; FRTG13/FRTG13 tubGAL80*;

tubGAL4/2x-UAS-eGFP. Mutant genotypes used were: *esg^{G66} (y,w, hsflp¹²²; FRT40A esg^{G66}/FRT40A tubGAL80; tubGAL4/2x-UAS-eGFP)*, *esg^{L2} (y,w, hsflp¹²²; FRT40A esg^{L2}/FRT40A tubGAL80; tubGAL4/2x-UAS-eGFP)*, *shg^{IG29} (y,w, hsflp¹²²; FRTG13 shg^{IG29}/FRTG13 tubGAL80; tubGAL4/2x-UAS-eGFP)*, *shg^{IH} (y,w, hsflp¹²²; FRTG13 shg^{IH}/FRTG13 tubGAL80; tubGAL4/2x-UAS-eGFP)*.

Immunostaining and microscopy

Testes were dissected and fixed in 2% PFA in PLP buffer (0.075 M lysine, 0.01 M sodium phosphate buffer pH 7.4) for 60 min at room temperature, rinsed for 30 min in PBS containing 0.3% Triton X-100 and 0.3% sodium deoxycholate, rinsed for 15 min in PBS containing 0.1% Triton X-100, blocked for 30 min in PBS containing 0.1% Triton X-100 and 0.3% bovine serum albumin, and immunostained with appropriate antibodies over-night at 4 °C. Testes were then washed for 60 min at room temperature in PBS containing 0.1% Triton X-100, and incubated with appropriate secondary antibodies at room temperature for 4 h. Testes were analysed with a Zeiss Axiovert 200 microscope and processed, and AxioVision (version 4.5; C. Zeiss) and Adobe Photoshop software. Analysis of FasIII-, TJ- and β -galactosidase-labelled testes were performed using a Leica TCS SP2 AOBS confocal microscope and LCS Lite 2.61.1537 software (Leica Microsystems).

Antibodies

Testes were stained using the following: rabbit anti- β -galactosidase (1:2,000) (Cappel), guinea-pig anti-TJ (1:3,000) (gift from D. Godt), mouse anti-BrdU (1:100) (BD Biosciences), rat-anti-BrdU (1:100) (Accurate Chemicals), rabbit anti-GFP (1:5,000) (Molecular Probes), and rabbit anti-phosphorylated histone H3 (1:200) (Upstate Biotechnologies). Mouse anti-Fasciclin III (7G10) (1:10) and rat anti-DE-cadherin (DCAD2) (1:20) were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies were diluted 1:500 (Molecular Probes). Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

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Figure 1. Somatic stem cell progeny contribute to the hub

a, Schematic diagram of the apical tip of the testis. Hub cells (red) contact SSCs (light grey) and GSCs (green). SSCs produce cyst cells (dark grey) that envelop developing germline cells (green). **b-f**, Immunofluorescence images of the apical tip of the adult testis from flies heat shocked to generate labelled dividing cells expressing nuclear β -galactosidase (β -gal). Testes were stained with antibodies to DE-cadherin or Fasciclin III (FasIII), β -gal, and Traffic Jam (TJ) or 4,6-diamidino-2-phenylindole (DAPI). **b**, One day after heat shock, three β -gal⁺ (arrowheads, green; second panel) and TJ⁺ somatic cells (red; fourth panel) are near the FasIII⁺ (blue; third panel) hub (asterisk). **c**, Five days after heat shock two β -gal⁺ SSCs (arrowheads, green; right panel) contact hub cells (outline/DE-cadherin, red). A β -gal⁺ SSC daughter cell (arrow) is also present. **d**, Lower magnification view of **c**. β -Gal⁺ somatic cells (arrows, green) are present along the length of the testis. **e**, Five days after heat shock, the hub (outline, DE-cadherin, red) contains two β -gal⁺ cells (arrowheads, green; right panel). **f**, Fifteen days after heat shock, two β -gal⁺ hub cells (green, arrowheads; second panel) co-stain with FasIII (blue, outline; third panel) and TJ (red; fourth panel). Scale bars: **b**, **d**, 20 μ m; **c**, **e**, **f**, 10 μ m.

Figure 2. BrdU-labelled cells become incorporated into the apical hub

a, b, d, Immunofluorescence images of testes from wild-type (*OregonR*) (**a, b**) or *updGAL4-UAS-gfp* (**d**) adult flies fed BrdU. **a**, Left panel: BrdU⁺ cells (green) surround the FasIII⁺ hub (red, outline). Two BrdU⁺ GSCs (asterisks) and a BrdU⁺ somatic cell (arrow) are adjacent to the apical hub (outline) after a 1-day chase. Right panel: no BrdU⁺ hub cells are detected. **b**, A BrdU⁺ hub cell (arrow) is present after a 3-day chase (left panel). FasIII (middle panel); BrdU (right panel). **c**, Hub cells stained with DE-cadherin (red; middle panel) and GFP (green; right panel) in *updGAL4-UAS-gfp* testis. **d**, A BrdU⁺ hub cell (arrow) is detected after a 7-day chase. The middle panel shows the GFP channel; the right panel shows the BrdU channel. Scale bars: 10 μ m.

Figure 3. Two populations of mitotically active somatic cells are present near the hub
a-d, Confocal images of *updGAL4-UAS-gfp* testes. **a, b**, Testes stained for Vasa (blue; middle panels), phospho-histone-H3 (pHH3, red; right panels) and GFP (green). Phospho-histone-H3⁺ SSCs (arrowheads) are observed near the hub. **c**, Testes stained for TJ (blue; middle panel), phospho-histone-H3 (green; right panel) and GFP (green; right panel). The hub is outlined. A mitotic CPC (arrow) surrounds a mitotic GSC (arrowhead). **d**, Testes stained with TJ (blue; middle panel), phosphohistone-H3 (red; right panel) and GFP (green). A mitotic CPC (arrows) one-cell distance from the hub (outline) is shown. **e**, Graph depicting marked somatic cell type frequency from all testes assayed at various time points. **f**, Graph depicting marked somatic cell type frequency from testes containing marked somatic cells at various time points. **g-i**, A testis 10 days after heat shock stained for β -gal⁺ (green; bottom panels), DE-cadherin (red) and DAPI (blue). A β -gal⁺ hub cell (**g**, indented arrowhead), β -gal⁺ SSC (**h, i**, arrowhead) and β -gal⁺ CPC (**i**, arrow) are shown. Z-section images (1 μ m) are denoted by depth in the lower left corner. Scale bars: 10 μ m.

Figure 4. Factors required for SSC maintenance and the SSC-hub cell transition

a, Ten days after heat shock a series of GFP⁺ late cyst cells (green, arrows) and a single labelled hub cell are present (arrowhead in **b**). GFP (green), DE-cadherin (red) and DAPI (blue) are shown. **b**, Higher magnification ($\times 63$) of the hub in **a** (outline, DE-cadherin, red) with a GFP⁺ hub cell (arrowhead, right panel). **c**, Five days after heat shock GFP⁺ germline and somatic cells are maintained near the DE-cadherin⁺ hub (red). Late GFP⁺ germline (arrowhead) and somatic cell (arrow) clones are present basally. **d**, Five days after heat shock GFP⁺ *shg*^{IG29} mutant late somatic cells are present (arrow), but no GFP⁺ clones are present near the DE-cadherin⁺ hub (red) at the apical tip. **e**, **f**, Fifteen days after heat shock GFP⁺ hub cells are present from wild-type (**e**) and *shg*^{IG29} (**f**) somatic clones (arrowheads). GFP⁺ *shg*^{IG29} hub cells display loss of DE-cadherin (red) at the hub interface (inset, DE-cadherin channel). **g**, **h**, Testes from 1-day-old *c587GAL4* (**g**) and *c587GAL4/UAS-shgRNAi* (**h**) males stained with DE-cadherin (red) and TJ (green). **i**, **j**, Testes from 15-day-old *c587GAL4* (**i**) and *c587GAL4/UAS-shgRNAi* (**j**) males. Note the loss of DE-cadherin (red) and TJ (green) stain in **j**. **k**, **l**, Fifteen days after heat shock distorted GFP⁺ hub cells are present from *esg*^{G66} (**k**) and *esg*^{L2} (**l**) mutant somatic clones (compare to **e**). The hub is marked with DE-cadherin (red). **m**, **n**, *esg*^{shof}/*CyO*; *osk/osk* (**m**) and *esg*^{shof}/*esg*^{shof}; *osk/osk* (**n**) testes stained for FasIII (red, insets) and TJ (green). Scale bars: **a**, **c**, **d**, 20 μ m; **e**-**n**, 10 μ m.