Supplemental Information

Conversion of quiescent niche cells to somatic stem cells causes ectopic niche formation in the Drosophila testis

Hétié, de Cuevas, and Matunis

Figure S1 (related to Fig. 1)

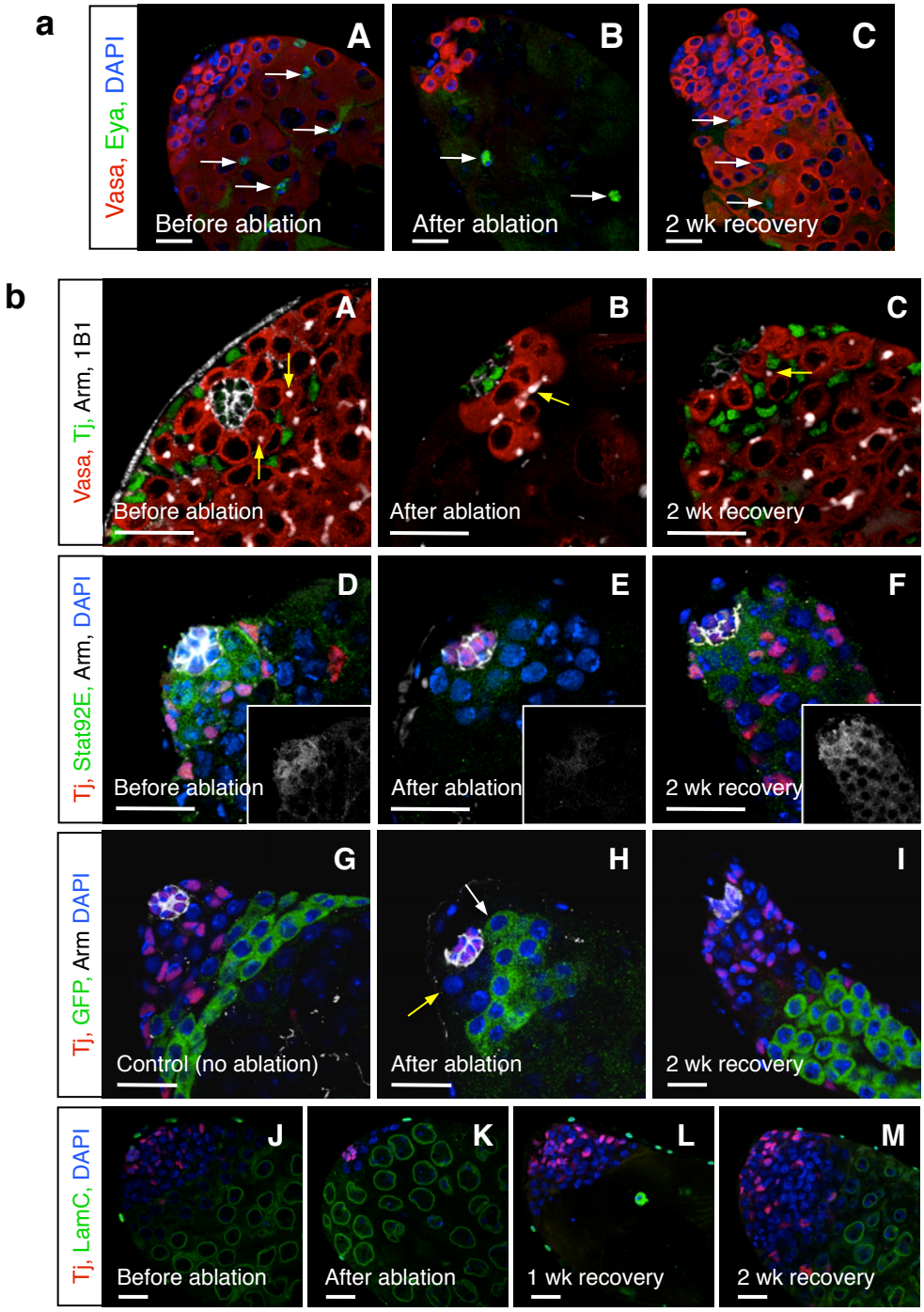

Figure S1, *Hétié et al.*

Figure S1 (*continued*)

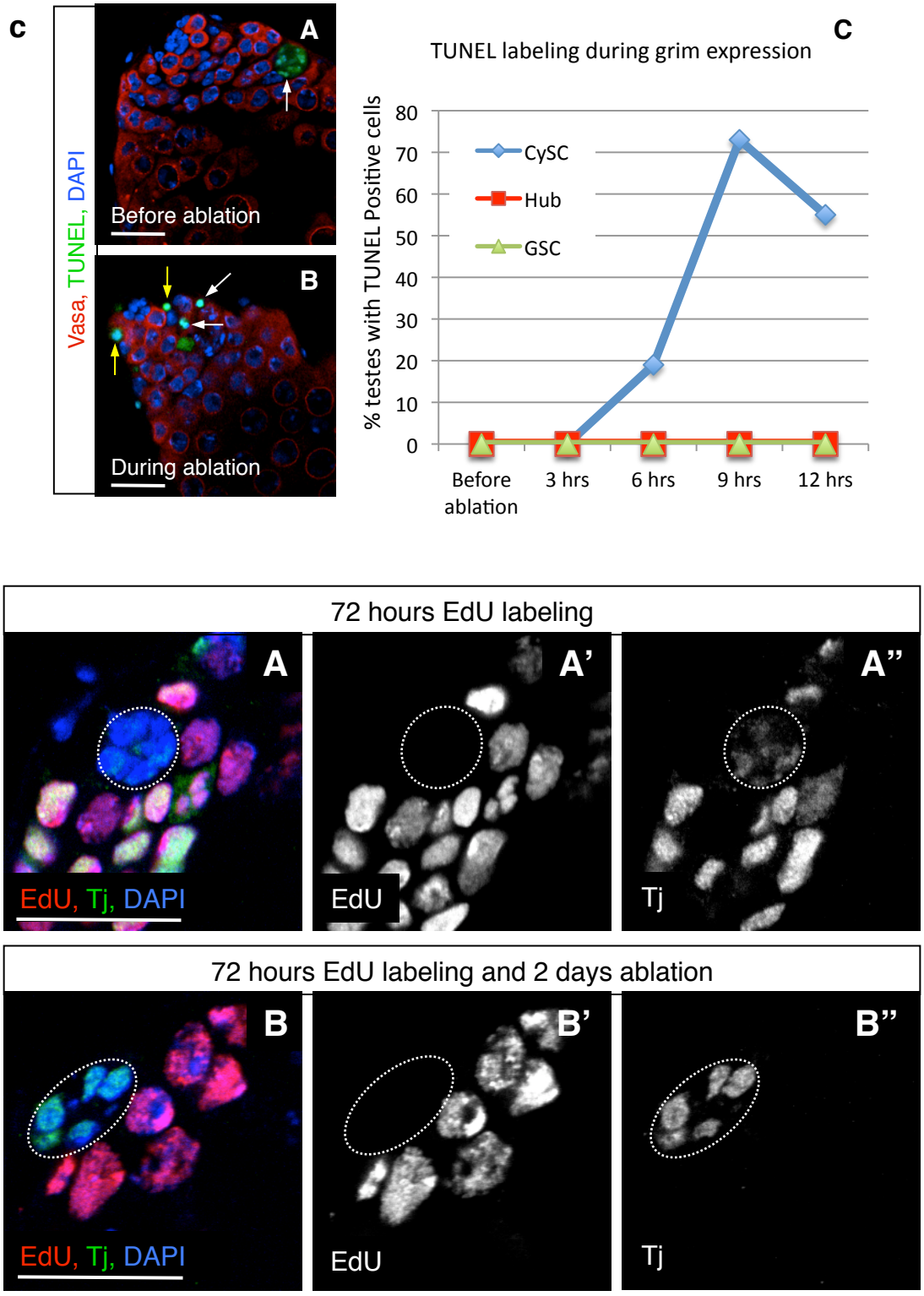

Figure S1, *Hétié et al.*

Figure S1, related to Fig. 1: Recovery of functional CySCs after complete genetic ablation of CySCs and early cyst cells in the adult testis.

a) Eya-positive late cyst cells remain after genetic ablation and recovery of CySCs and early cyst cells. (A-C) Single confocal sections through the apex of *c587-Gal4>grim* testes immunostained with anti-Vasa (red; germ cells) and anti-Eyes absent (Eya, green; late cyst cells; arrows). Nuclei are counterstained with DAPI (blue). (A) Eya-positive cyst cells associate with spermatocytes before ablation and (B) remain after CySC ablation. (C) After CySC ablation and 2 weeks of recovery, testes that regain CySCs contain Eya-positive cyst cells associated with spermatocytes. Scale bars, 20 μm.

b) GSCs and their descendants are maintained after genetic ablation and recovery of CySCs and early cyst cells. (A-M) Single confocal sections through the testis apex. Nuclei are counterstained with DAPI (D-M, blue). (A-C) *c587-Gal4>grim* testes are immunostained with anti-Vasa (red; germ cells); anti-Tj (green; hub cells, CySCs, and early cyst cells); anti-Arm (white; hub cells); and anti-Hu li tai shao (Hts/1B1, white; fusomes). (A) Before CySC ablation, testes appear wild type: GSCs containing round fusomes (arrows) surround the hub. (B) After CySC ablation, early germ cells (GSCs and/or spermatogonia with elongated or branched fusomes; arrow) remain in all testes (n = 784). (C) After CySC ablation and 2 weeks of recovery, in all testes that regain CySCs, the hub is surrounded by GSCs containing round fusomes (arrow) (n = 40). (D-F) *c587-Gal4>grim* testes immunostained with anti-Tj (red), anti-Stat92E (green), and anti-Arm (white). Insets show Stat92E only. (D) Before CySC ablation, Stat92E is expressed at high levels in GSCs and CySCs (n = 40). (E) After CySC ablation, Stat92E expression is reduced (n = 33). (F) After CySC ablation and 2 weeks of recovery, Stat92E expression is restored in testes that regain CySCs (n = 40). (G-I) Testes from control *c587-Gal4, bam-GFP* (G) or experimental *c587-Gal4>grim, bam-GFP* (H-I) adult flies were immunostained with anti-Tj (red), anti-GFP (green), and anti-Arm (white). GFP expression is driven by the *bag of marbles (bam)* promoter and marks clusters of interconnected spermatogonia containing 4, 8, or 16 cells (Gonczy et al., 1997; McKearin and Spradling, 1990). (G) Control testes in CySC ablation conditions resemble wild-type testes: they contain *bam*-GFP-expressing cells away from the hub. (H) After CySC ablation, GFP-negative cells (yellow arrow) contact the hub in all testes (n = 108); in a few testes, *bam*-GFP-expressing cells (white arrow) also contact the hub (n = 3/108). (I) After CySC ablation and 2 weeks of recovery, *bam*-GFP-expressing cells are found in all testes that regain CySCs, but they do not contact the hub (n = 37). (J-M) *c587-Gal4>grim* testes immunostained with anti-Tj (red) and anti-Lamin C (LamC; green), which marks the nuclear envelope in spermatocytes (Matsui et al., 2011). (J) Before CySC ablation, LamC-enriched spermatocytes are found in all testes (n = 41). (K) After CySC ablation, spermatocytes remain in 98% of testes (n = 128/130). (L) After ablation and one week of recovery, LamC-expressing spermatocytes remained in only 3% of testes (n = 3/101). (M) After 2 weeks of recovery, 53% of testes (n = 41/78) that regain CySCs also regain LamC-expressing spermatocytes. Scale bars, 20 μm.

c) *grim* induction in CySCs and early cyst cells induces apoptosis in these cells. (A-B) Single confocal sections through the apex of *c587-Gal4>grim* testes labeled with anti-

Vasa (red; germ cells) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, green) to visualize apoptotic cells. Nuclei are counterstained with DAPI (blue). (A) Before *grim* induction, testes contain TUNEL-positive cyst cells and spermatogonia (arrow) (Brawley and Matunis, 2004; Yacobi-Sharon et al., 2013), but no TUNEL-positive cells are found near the hub where GSCs and CySCs reside. (B) After 6 hours of *grim* induction, testes contain TUNEL-positive CySCs (yellow arrows), cyst cells (white arrows), and spermatogonia. No TUNEL-positive GSCs or hub cells were detected in this experiment. Scale bars, 20 μm. (C) Line graph showing the percentage of *c587-Gal4>grim* testes with TUNEL-positive cells before and during *grim* induction. After 3 hours of *grim* induction, no TUNEL-positive CySCs, GSCs, or hub cells were detected. After 6 or more hours of *grim* induction, the percentage of testes with TUNEL-positive CySCs increased dramatically. N = 20-22 testes per time point.

d) EdU-labeled CySCs do not enter the hub or convert into hub cells during genetic ablation of CySCs. (A,B) Single confocal sections through the apex of *c587-Gal4>grim* testes stained for the S-phase marker EdU (red) and anti-Tj (green; hub cells, CySCs, and early cyst cells); nuclei were counterstained with DAPI (blue). Adult flies were fed EdU for 72 hours at 18˚C; half were dissected immediately (A), and the rest were subjected to CySC ablation (2 days at 31˚C) prior to dissection (B). (A-A") After 72 hours of EdU labeling, EdU was incorporated into germ cells (Tj-negative) and CySC lineage cells (Tj-positive), but EdU was not detected in the hub. (B-B") After 72 hours of EdU labeling followed by CySC ablation, EdU was still detected in germ cells, but EdU was not detected in the hub (n = 25 testes). Scale bars, 20 μm.

Figure S2 (related to Fig. 2)

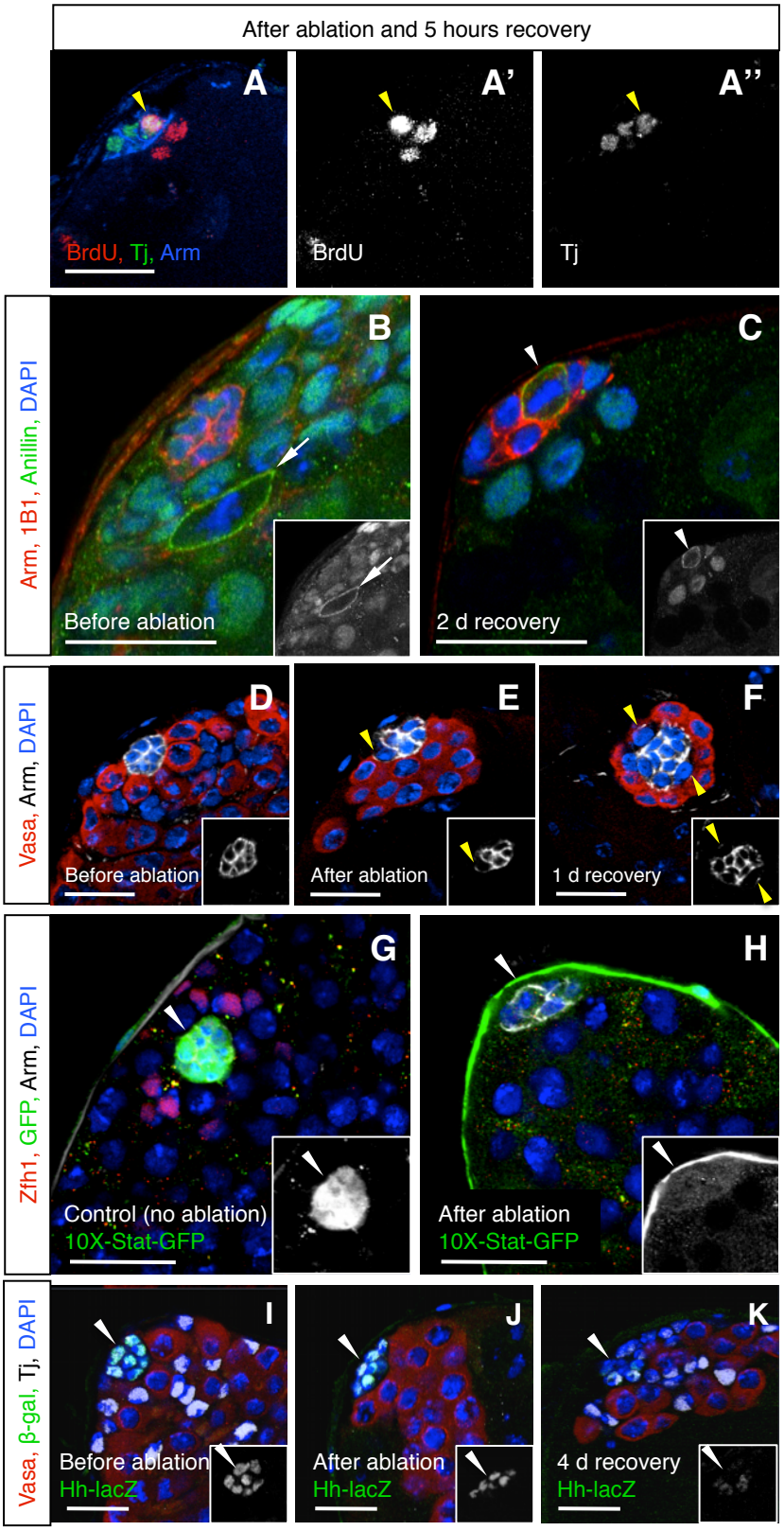

Figure S2, *Hétié et al.*

Figure S2, related to Fig. 2: After complete genetic ablation of CySCs, hub cells exit quiescence and convert into CySC-like cells.

(A-K) Single confocal sections through the apex of *c587-Gal4>grim* testes (except G, which is *c587-Gal4* alone) immunostained as indicated. Nuclei are counterstained with DAPI (B-K, blue). (A-A'') Testis immunostained with anti-Bromodeoxyuridine (BrdU, red), anti-Tj (green; hub cells, CySCs, and early cyst cells), and anti-Arm (blue; hub cells). BrdU is a thymidine analog that incorporates into S-phase cells. After CySC ablation, flies recovered at 18˚C for 5 hours on BrdU prior to dissection. BrdU was found in hub cells in 8% of testes (n = 7/89) (one shown, arrowhead) as well as in some germ cells (Tj-negative) outside the hub. After 24 hours of recovery with BrdU labeling, BrdU is found in hub cells in 31% of testes (n = 21/68) (not shown). (B-C) Testes immunostained with anti-Arm (red; hub cells), anti-Hts/1B1 (red; fusomes), and anti-Anillin (green and insets). (B) Before CySC ablation, Anillin is expressed in actively cycling cells, where it is located in the nucleus during interphase and at the cell cortex during mitosis (arrow) (Field and Alberts, 1995). (C) After CySC ablation and 2 days of recovery, Anillin is found at the cortex of mitotic hub cells (one shown, arrowhead). (D-F) Testes immunostained with anti-Vasa (red; germ cells) and anti-Arm (white and insets). (D) Before ablation, Arm is located between hub cells and at hub cell-GSC interfaces. (E) After ablation and (F) after one day of recovery, Arm is reduced in some hub cells (arrowheads). (G-H) Testes immunostained with anti-Zfh1 (red), anti-GFP (green and insets), and anti-Arm (white). GFP expression is driven by the 10X-Stat-GFP reporter, which reflects the level of Janus kinase-Signal transducer and activator (JAK-STAT) signaling activity in the hub (Bach et al., 2007). This reporter is also expressed in the testis sheath, particularly at higher temperatures. (G) Control testes (*c587-Gal4* alone) in CySC ablation conditions resemble wild-type testes: GFP is expressed at high levels in the hub (arrowheads). (H) After CySC ablation, GFP expression decreases in the hub (arrowheads). (I-K) Testes immunostained with anti-Vasa (red), anti-β-Galactosidase (β-Gal, green and insets), and anti-Tj (white). β-Gal expression is driven by the *hedgehog* promoter (*hh-lacZ*). (I) Before CySC ablation and (J) after CySC ablation, β-Gal is expressed strongly in hub cells (arrowheads). (K) After CySC ablation and 4 days of recovery, β-Gal expression in hub cells is reduced (arrowheads). Scale bars, 20 μm.

Figure S3 (related to Fig. 4)

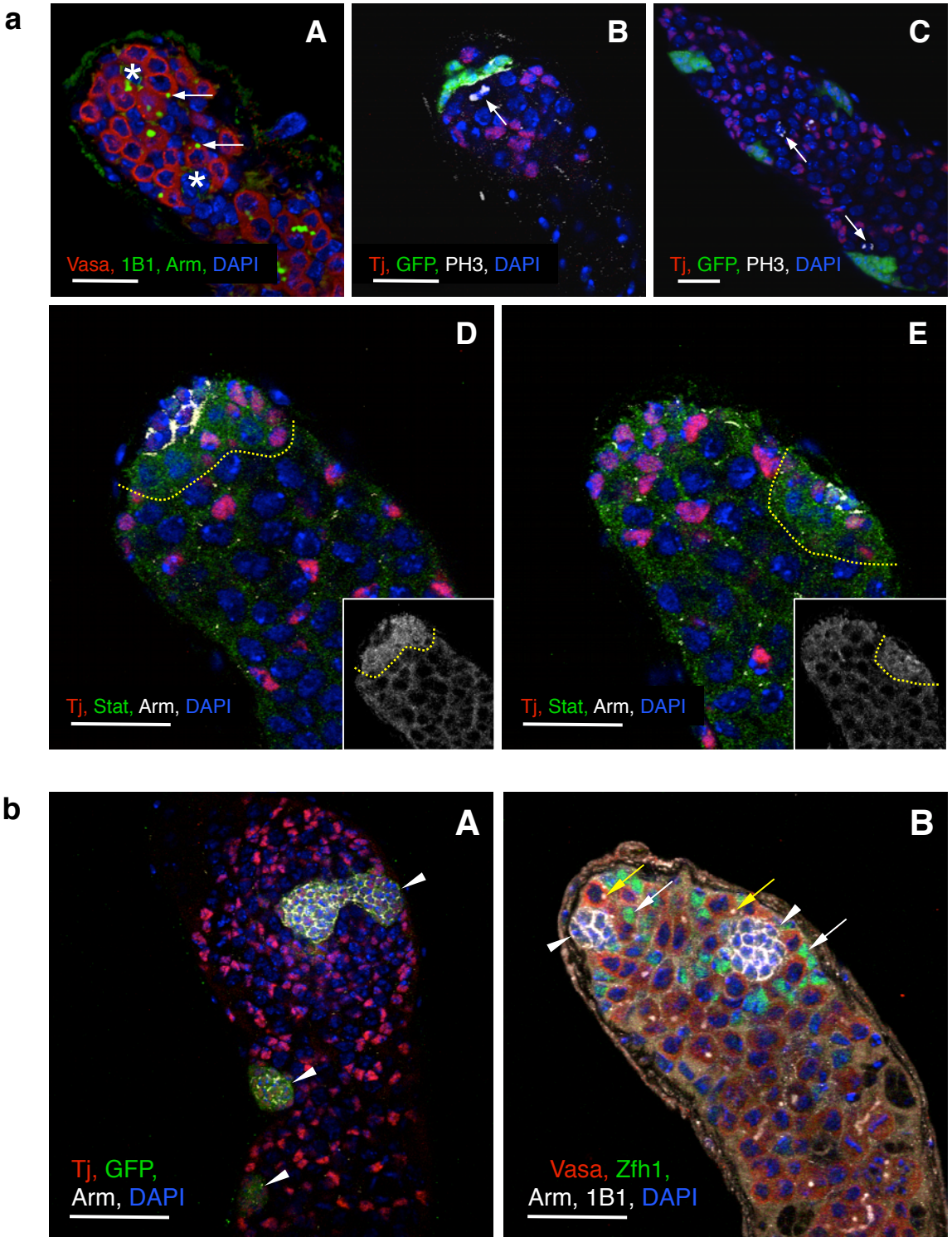

Figure S3, *Hétié et al.*

Figure S3, related to Fig. 4: Hub cell conversion to CySCs promotes the formation of ectopic functional stem cell niches.

a) Ectopic niches support mitotic stem cells. (A-E) Single confocal sections through the apex of *c587-Gal4>grim* testes, after CySC ablation and two weeks of recovery, immunostained as indicated. Nuclei are counterstained with DAPI (blue). All testes shown here contain multiple ectopic hubs, some outside the plane of focus. (A) Testis immunostained with anti-Vasa (red; germ cells), anti-Hts/1B1 (green; fusomes), and anti-Arm (green; hub cells). Germ cells with single round fusomes (arrows) are located next to the two ectopic hubs (asterisks) visible in this focal plane. Another hub is located in a different focal plane (not shown). (B-C) Testes immunostained with anti-Tj (red; hub cells, CySCs, and early cyst cells), anti-GFP (GFP, green; hub cells), anti-Arm (white; hub cells), and mitotic marker anti-PH3 (white). GFP expression is driven by the Fas3 promoter and marks hub cells. Single mitotic germ cells (Tj-negative/PH3-positive; arrows) are found near (B) an apically located hub and (C) hubs that are displaced from the apex. The apically located hub in (B) is irregularly shaped. (D-E) Two different focal planes from the same testis immunostained with anti-Tj (red), anti-Stat92E (green and insets), and anti-Arm (white). Stat92E is enriched in cells (outlined) surrounding (D) the apically located hub and (E) an ectopic hub. Scale bars, 20 μm.

b) Ectopic CycD-Cdk4 expression in the hub causes ectopic niche formation. (A-B) Single confocal sections through the apex of *E132-Gal4/Y; UAS-CycD,UAS-Cdk4/Fas3-GFP; tub-Gal80[ts]/+* testes, after two weeks at 31˚C and two weeks of recovery at 18˚C, immunostained as indicated. Nuclei are counterstained with DAPI (blue). (A) Testis immunostained with anti-Tj (red; hub cells, CySCs, and early cyst cells), anti-Arm (white; hub cells), and anti-GFP (green; hub cells). GFP expression is driven by the Fas3 promoter and marks hub cells. After ectopic CycD-Cdk4 expression in hub cells, three hubs (arrowheads) are visible in this testis; the most apical hub has a dumb-bell shape. (B) Testis immunostained with anti-Vasa (red; germ cells), anti-Hts/1B1 (white; fusomes), anti-Arm (white; hub cells), and anti-Zfh1 (green; CySCs). Zfh1-positive cells (white arrows) and germ cells with single round fusomes (yellow arrows) are located next to the apical and ectopic hubs. Scale bars, 20 μm.

Table S1 (related to Figure 1)

a. Ectopic *grim* expression using two independent CySC lineage Gal4 drivers ablates all CySCs.

Genotype	Testes with complete CySC ablation	Testes that regained CySCs after recovery	Testes with ectopic niches/testes that regained CySCs
c587-Gal4, UAS-Grim[a]	100% (813/813)[c]	65% (328/506) [d]	42% (60/144) [d]
Tj-Gal4, UAS-Grim[b]	100% (19/19)[c]	71% (10/14) [e]	50% (5/10) [e]

[a] genotype*: c587-Gal4/Y; UAS-Grim/+; tub-Gal80^{ts}/+*
[b] genotype*: w/Y; Tj-Gal4/UAS-grim; tub-Gal80^{ts}/+*
[c] raised at 18˚C, transferred to 31˚C for 2 days
[d] raised at 18˚C, transferred to 31˚C for 2 days, returned to 18˚C for 2 weeks
[e] raised at 18˚C, transferred to 31˚C for 2 days, returned to 18˚C for 3 weeks

b. Ectopic *grim* expression in the CySC lineage induces apoptosis in CySCs but not in hub cells or GSCs.

Hours of induction @ 31ºC			0	3	6	9	12
% Testes[a] with TUNEL-positive:	CySCs[b]	experimental males[c]	0	0	19	73	55
		control males[d]	0	0	0	0	0
	Hub cells[b]	experimental males[c]	0	0	0	0	0
		control males[d]	0	0	0	0	0
	GSCs[b]	experimental males[c]	0	0	0	0	0
		control males[d]	0	0	0	0	0
	Cyst cells	experimental males[c]	90	100	100	100	100
		control males[d]	90[e]	90	85	90	85
	Spermatogonia	experimental males[c]	100	43	57	90	95
		control males[d]	90[e]	85	60	60	60

[a] 20-22 testes were analyzed for each sample.
[b] data shown graphically in Fig. S1c, panel C.
[c] genotype: *c587-Gal4/Y; UAS-Grim/+; tub-Gal80^{ts}/+*
[d] genotype: *c587-Gal4/Y; +; UAS-GFP/tub-Gal80^{ts}*
[e] TUNEL-positive cyst cells and spermatogonia, frequently observed in control testes as expected (Brawley and Matunis, 2004; Yacobi-Sharon et al., 2013) provide an internal control for TUNEL labeling.

Table S2 (related to Figure 2)

a. Hub cells transiently express the mitotic marker phospho-histone H3 (PH3) during recovery from CySC ablation.

Days after CySC ablation	Testes with PH3-positive hub cells[a]
0	0% (0/158)
1	7% (12/174)
2	10% (13/133)
3	10% (17/173)
4	6% (8/129)
5	7% (7/102)
6	<1% (1/131)
7	0% (0/147)

[a] genotype: *c587-Gal4/Y; UAS-Grim/+; tub-Gal80ts/+*

b. M5-4 is expressed in the CySC lineage during recovery[c] from CySC ablation.

	Ablation condition	Testes with CySCs after ablation	Testes with CySCs after recovery	Testes with M5-4(+) CySCs/testes with CySCs after recovery
Experimental flies[a]	1d. 30ºC[d]	39% (22/56)	38% (12/32)	67% (8/12)
	1d. 31ºC	15% (6/39)	38% (18/48)	78% (14/18)
	2d. 31ºC	0% (0/33)	69% (24/35)	100% (24/24)
Control flies[b]	2d. 31ºC	100% (31/31)	100% (33/33)	0% (33/33)

[a] genotype: *c587-Gal4; UAS-Grim/M5-4-lacZ; tub-Gal80ts/+*

[b] genotype (processed in parallel to control for the effect of ablation conditions on M5-4 expression): *c587-Gal4; SM6B/M5-4-lacZ; tub-Gal80ts/+*

[c] recovery for 5-7 days

[d] This ablation condition was used in a prior study, but hub cell conversion was not reported (Lim and Fuller, 2013). Under these conditions similar numbers of testes contain CySCs before and after ablation (39 and 38%, respectively). Since M5-4 is expressed in all testes regaining CySCs after complete ablation (2d, 31˚C) but not in controls processed in parallel, the appearance of M5-4 in most (67 or 78%) testes recovering from milder ablation conditions reveals that hub cell conversion is occurring; the remaining testes must be regaining CySCs from CySCs that were not ablated. In this experiment, CySCs arising from conversion can be distinguished only by their co-expression of M5-4 and Tj, which explains why they were not reported previously.

Table S3 (related to Figure 2)

a. Hub cell conversion does not occur in testes regaining CySCs during recovery from nutrient deprivation.

Genotype	Feeding protocol	Total testes	Testes with β-Gal+ hub cells	Testes with β-Gal+ CySC clones[c]
E132-Gal4[a]	fed 25 d.	260	242	8
	starved 20 d., fed 5 d.	117	115	0
Fas3-GS-Gal4[b]	starved 20 d., fed 5 d.	467	n.d.	1

[a] genotype: *E132-Gal4/Y; UAS-FLP/+; Act5c>stop>lacZ/tub-gal80^{ts}*
[b] genotype: *+/Y; Fas3-GS-Gal4/UAS-FLP; Act5c>stop>lacZ/+* or +/Y; *Fas3-GS-Gal4/+; Act5c>stop>lacZ/UAS-FLP*
[c] Because the drivers used for this experiment are expressed outside the hub during development, we surmise that "background" clones in control testes arise from leakiness in the marking system. However, we cannot rule out that hub cell conversion occurs at a low level under normal conditions. In either case, hub cell conversion does not occur at higher levels during recovery from partial loss of CySCs.

b. Hub cell conversion does not occur during normal aging.

Genotype	Age at dissection	Total testes	Testes with β-Gal+ hub cells	Testes with β-Gal+ CySC clones[c]
E132-Gal4[a]	< 1 wk.	81	81	2
	3-4 wk.	109	109	1
Fas3-GS-Gal4[b]	< 1 wk.	45	36	0
	5-6 wk.	30	30	0

[a] genotype: *E132-Gal4/Y; UAS-FLP/+; Act5c>stop>lacZ/tub-gal80^{ts}*
[b] genotype: *+/Y; Fas3-GS-Gal4/UAS-FLP; Act5c>stop>lacZ/+* or +/Y; *Fas3-GS-Gal4/+; Act5c>stop>lacZ/UAS-FLP*
[c] See footnote to Table S3a.

Table S4 (related to Figure 3). Overexpression of CycD-Cdk4 in the hub is sufficient to cause hub cell-to-CySC conversion[a].

Gentotype	Temperature protocol	Testes with CySC clones[d]
Hub lineage tracing with CycD-Cdk4[b]	18˚C 0 d (newly eclosed)	14% (19/132)
	18˚C 2 wks.	26% (28/109)
	31˚C 1 wk., 18˚C 1 wk.	55% (55/100)
Hub lineage tracing without CycD-Cdk4[c]	18˚C 0 d (newly eclosed)	3% (4/125)
	18˚C 2 wks.	5% (7/135)
	31˚C 1 wk., 18˚C 1 wk.	8% (15/178)

[a] The data in this table are presented graphically in Fig. 3G.
[b] genotype: *E132-Gal4/Y; UAS-CycD, UAS-Cdk4/+; UAS-Flp/Act5c>stop>lacZ*
[c] gemotype*: E132-Gal4/Y; +; UAS-Flp/Act5c>stop>lacZ*
[d] Because the driver used for this experiment is expressed outside the hub during development, we surmise that "background" clones in testes from uninduced and control males arise from leakiness in the marking system. However, we cannot rule out that hub cell conversion occurs at a low level under normal conditions and is accelerated by hub cell re-entry into the cell cycle.

Table S5 (related to Figure 4). Overexpression of CycD-Cdk4 causes ectopic hub formation in adult testes.

Genotype	Temperature protocol	% testes with ectopic hubs
GFP-marked hub with CycD-Cdk4[a]	newly eclosed	1% (5/384)
	31˚C 2 wk., 18˚C 2 wk.	7% (13/180) ***
	18˚C 4 wk.	1% (4/544)
GFP-marked hub without CycD-Cdk4[b]	newly eclosed	0% (0/273)
	31˚C 2 wk., 18˚C 2 wk.	0% (0/137)

[a] genotype: *E132-Gal4/Y; Fas3-GFP/UAS-CycD, UAS-Cdk4; tub-Gal80^{ts}/+*
[b] genotype: *E132-Gal4/Y; Fas3-GFP/+; tub-Gal80^{ts}*
*** $P < 0.0001$ compared to controls lacking CycD-Cdk4.

Supplemental Experimental Procedures

Fly stocks

The following transgenes were used: c587-Gal4, from A. Spradling (Kai and Spradling, 2003); E132-Gal4 (also called upd-Gal4), from H. Sun (Brand and Perrimon, 1993); Tj-Gal4, from the Drosophila Genetic Resource Center (Kyoto), no. 104055; UAS-Grim, from J. Abrams (Chen et al., 1996); M5-4-lacZ, from S. DiNardo (Gonczy and DiNardo, 1996); bam promoter-GFP, from D. McKearin (Chen and McKearin, 2003); UAS-CycD, UAS-Cdk4, from B. Edgar (Datar et al., 2000); hh-lacZ, from D. Drummond-Barbosa (Forbes et al., 1996); and Fas3-GS-Gal4, from B. Ohlstein (Roman et al., 2001). All other stocks were from the Bloomington Drosophila Stock Center.

Immunofluorescence microscopy

Testes were dissected, fixed, and immunostained as described (Matunis et al., 1997). The following antibodies were used: rabbit anti-Vasa d-260 (Santa Cruz Biotechnology, 1:400 dilution); rabbit anti-GFP (Torrey Pines Biolabs, 1:10,000); rabbit anti-Anillin from C. Field, 1:1000) (Field and Alberts, 1995); affinity-purified rabbit anti-Stat92E (from E. Bach, 1:100) (Flaherty et al., 2010); guinea pig anti-Traffic jam (from D. Godt, 1:10,000) (Li et al., 2003); rabbit anti-Zfh1 (from R. Lehmann, 1:10,000); mouse anti-phospho-Histone H3 (Ser10) (Cell Signaling, 1:200); rabbit anti-phospho-Histone H3 (Ser10) (Upstate/Millipore, 1:200); rat anti-bromodeoxyuridine (MCA2060) (AbD Serotec, 1:40); mouse anti-β-galactosidase (Z3783) (Promega, 1:1000); and mouse monoclonal antibody 1B1 (1:50), mouse anti-Armadillo N2 7A1 (1:50), mouse anti-Bam (1:1500, concentrated form), rat anti-CadherinN DN-EX #8 (1:20), mouse anti-Eya 10H6 (1:10), and mouse anti-Lamin C LC28.26 (1:20), all from Developmental Studies Hybridoma Bank. Alexa Fluor 405, 488, 568, and 633 secondary antibodies (Molecular Probes/Invitrogen) were used at a 1:200 or 1:400 dilution. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) at 1ug/ml. Testes stained with anti-Eya or anti-Stat92E were incubated in primary antibody for 3 days or 2 days respectively at 4˚C. Bam-expressing spermatogonia were identified by staining either with anti-Bam antisera or with anti-GFP antisera in *bam promoter-GFP* testes. Testes were imaged using a Zeiss LSM 5 Pascal or Zeiss LSM 510 Meta laser scanning microscope. Images were processed using Zeiss LSM or Zen software and assembled using Microsoft PowerPoint.

Apoptosis detection

Cells undergoing apoptosis were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Chemicon International) as described (Sheng et al., 2009). TUNEL-positive CySCs and cyst cells were identified by absence of Vasa staining and the position of their nuclei (less or greater than two cell diameters from the hub, respectively). TUNEL-positive, Vasa-positive cells (germ cells) were not seen within two cell diameters of the hub; TUNEL-positive clusters of germ cells greater than two cell diameters from the hub were scored as dying spermatogonia.

β-galactosidase detection

β-galactosidase was detected either by immunofluorescence (described above) or by X-gal staining as described (Gonczy et al., 1992) with the following modifications: testes

were incubated in X-gal solution for 2-3 hours and were mounted in 80% glycerol plus 5 mM each of potassium ferri- and potassium ferro-cyanide. X-gal-stained testes were scored on a Zeiss Axiovert or Olympus BX51 light microscope, or on a Zeiss Stemi SV6 stereomicroscope.

Drug feeding

For in vivo labeling of cells undergoing S-phase, flies were placed in vials containing filter paper soaked with 100 μl of 2.5 mM BrdU or 10 mM EdU in apple juice plus 5 μl green food coloring (McCormick). After feeding, only flies with green-dyed guts were dissected; the rest were discarded. BrdU and EdU were detected as described (Brawley and Matunis, 2004; Leatherman and DiNardo, 2010). For *Fas3-GS-Gal4* induction, flies were placed in vials containing filter paper soaked with 125 μl of 1 mg/ml RU486 in apple juice plus 5 μl green food coloring and were transferred to fresh drug/apple juice vials daily.

Lineage tracing of hub cells

In the following genotypes, expression or activation of Gal4 causes the permanent expression of β-galactosidase in hub cells and their descendents. Marked cells (β-galactosidase-positive) were detected by X-gal staining. Testes with four or more marked CySC lineage cells in the apical quarter of the testis were scored as positive for CySC clones (typically over 20 marked CySC lineage cells were present in this region). To trace hub cells after *CycD-Cdk4* induction, *E132-Gal4; UAS-CycD, UAS-Cdk4/+; Act5c>stop>lacZ/UAS-FLP* males were shifted to 31˚C for 7 days and returned to 18˚C for 7 days to allow CySC clones to develop; uninduced controls were aged at 18˚C for 14 days. *E132-Gal4; Act5c>stop>lacZ/UAS-FLP* males lacking ectopic *CycD-Cdk4* were processed in parallel to control for the effects of age and temperature. To trace hub cells in otherwise wild-type testes, *E132-Gal4; UAS-FLP/+; Act5c>stop>lacZ/tub-gal80^{ts}* males were raised at 25˚C, or raised at 18˚C and then shifted to 31˚C for 2 days to boost Gal4 expression; *Fas3-GS-Gal4/UAS-FLP; Act5c>stop>lacZ/+* or *Fas3-GS-Gal4/+; Act5c>stop>lacZ/UAS-FLP* males, which express a drug-inducible version of Gal4 that is active only when bound to RU486 (Roman et al., 2001), were raised at 25˚C and fed 1 mg/ml RU486 in apple juice for 2-4 days.

Supplemental References

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