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## Escargot restricts niche cell to stem cell conversion in the *Drosophila* testis

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### Summary

Stem cells reside within specialized microenvironments, or niches, that control many aspects of stem cell behaviour. Somatic hub cells in the *Drosophila* testis regulate the behaviour of cyst stem cells (CySCs) and germline stem cells (GSCs) and are a primary component of the testis stem cell

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#### Author Contributions

J.V., S.S., L.P.R., M.F. and L.J. planned experiments. J.V., S.S., L.P.R., A.A. and L.J. performed experiments and analysed data. G.H. identified, mapped and characterized the *shof* allele. M.L.C. generated reagents used in the study. J.V., S.S., G.H., L.P.R., M.L.C., A.A., T.H., M.F. and L.J. wrote and edited the manuscript.

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niche. The *shutoff* (*shof*) mutation, characterized by premature loss of GSCs and CySCs, was mapped to a locus encoding the evolutionarily conserved transcription factor Escargot (Esg). Hub cells depleted of Esg acquire CySC characteristics and differentiate as cyst cells, resulting in complete loss of hub cells and eventually, CySCs and GSCs, similar to the *shof* mutant phenotype. We identified Esg-interacting proteins and demonstrate an interaction between Esg and the co-repressor C-terminal binding protein (CtBP), which was also required for maintenance of hub cell fate. Our results indicate that niche cells can acquire stem cell properties upon removal of a single transcription factor *in vivo*.

## Keywords

stem cell; niche; germline; *Drosophila*; Escargot; CtBP

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## Introduction

Adult stem cells possess the capacity to self-renew and generate differentiated progeny that contribute to tissue maintenance. The capacity to undergo self-renewing divisions is regulated by intrinsic cellular determinants and requires an instructive local microenvironment, also known as the stem cell ‘niche’ (Schofield, 1978). Stem cell niches are comprised of a variety of components including support cells, soluble signalling factors, adhesion molecules, extracellular matrix, and circulatory or neuronal inputs (reviewed in Jones and Wagers, 2008). A primary role of the niche is to maintain the strict balance between stem and progenitor cells during tissue homeostasis; however, niche components must also coordinate an appropriate stem cell response to acute environmental changes and/or tissue damage.

*Drosophila melanogaster* has provided a genetically tractable model organism for addressing how communication between stem cells and the niche is regulated *in vivo*. At the tip of the *Drosophila* testis, approximately ten somatic hub cells are in direct physical contact with two stem cell populations: germline stem cells (GSCs) and somatic cyst stem cells (CySCs) (Figure 1A) (reviewed in Fuller, 1993). Hub cells secrete factors, such as the ligand Unpaired (Upd), which activates the JAK-STAT pathway in adjacent GSCs and CySCs, to regulate stem cell behaviour (Kiger et al., 2001b; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). In addition to the JAK-STAT pathway, Hh (Amoyel et al., 2013; Michel et al., 2012; Zhang et al., 2013) and BMP (Kawase et al., 2004; Leatherman and Dinardo, 2010; Michel et al., 2011; Shivdasani and Ingham, 2003; Zheng et al., 2011) signalling also play an important role in regulating stem cell behaviour within the testis stem cell niche.

CySCs are anchored at the tip of the testis, adjacent to hub cells, where they divide to self-renew and generate cyst cells that will differentiate in concert with the germ cells they surround (Cheng et al., 2011; Gönczy and DiNardo, 1996; Issigonis et al., 2009). JAK-STAT signalling acts intrinsically within CySCs to regulate CySC self-renewal and maintenance. In addition, activation of Stat92E, the single Stat orthologue in *Drosophila*, in CySCs is also important for regulating self-renewal of adjacent GSCs in a non-autonomous

manner (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Putative Stat92E targets have been identified in cyst cells, such as *zfh-1* and *chinmo*, that act intrinsically to regulate CySC behaviour and are sufficient to direct GSC proliferation (Flaherty et al., 2010; Leatherman and Dinardo, 2008). Signaling via the BMP pathway is also an important mechanism by which the CySCs can regulate germ cell behaviour (Shivdasani 2003; Kawase 2004; Leatherman and Dinardo, 2010). Results from lineage-tracing analysis also suggested that CySCs are capable of contributing cells to the hub; however, questions remain regarding the frequency of contribution and to what degree it is influenced by genetic variation (Dinardo et al., 2011; Voog et al., 2008).

GSCs are in direct contact with hub cells via adherens junctions and primarily undergo asymmetric divisions, with the mitotic spindle orientated orthogonal to hub cells, as a mechanism to ensure GSC self-renewal and continual production of gametes (Inaba et al., 2010; Yamashita et al., 2003). However, in contrast to CySCs, activation of Stat92E within GSCs appears to be important for regulating hub cell-GSC adhesion, rather than proliferation (Leatherman and Dinardo, 2010). Taken together, these data highlight the intricate relationship between the hub, CySCs, and GSCs and underscore the critical role that CySCs play within the stem cell niche.

Recent work has demonstrated that fully differentiated cells are capable of being “reprogrammed” back to a pluripotent stem cell-like state upon the addition of defined factors (Papp and Plath, 2013). Direct conversion between differentiated cell types, without passing through a pluripotent state, has also been reported, highlighting a previously underappreciated cellular plasticity (reviewed in Graf, 2011). While these advances are promising for regenerative medicine, little is understood about how the stem cell niche can influence reprogramming and de-differentiation or trans-differentiation decisions. Here we show in the *Drosophila* testis that niche cells can acquire somatic stem cell properties upon removal of a single transcription factor *in vivo*, underscoring the importance of understanding the mechanisms utilized to balance cell fates within the stem cell niche.

## Results

### ***shutoff* is an allele of *escargot***

During the course of a genetic screen, a recessive, loss-of-function allele, named *shutoff* (*shof*), was recovered that resulted in premature and progressive loss of early male germ cells in testes evident by phase contrast microscopy (Figure 1B, F). Early germ cell loss was confirmed by examining the expression of an enhancer trap line that marks early germ cells, in combination with the germ cell marker Vasa (Figure 1C–C'', G–G''). Similarly, staining for the early cyst cell markers Zfh-1 and Traffic jam (TJ) revealed loss of early somatic CySCs and cyst cells in the testis (Figure 1E–E'', I–I'') (Leatherman and Dinardo, 2008; Li et al., 2003). Loss of stem cells appeared to be due to direct differentiation, as early somatic and germline cells differentiated at the apical tip of mutant testes (Figure 1F,G,I, Figure S3), and excessive apoptosis during development was not observed (Figure 2K–L).

Genetic recombination and mapping with deficiency chromosomes revealed that *shof* was likely an allele of *escargot* (*esg*), a member of the Snail family of transcriptional repressors

(Nieto, 2002). Previous studies demonstrated that *esg* is one of the first, sexually dimorphic markers expressed in *Drosophila* (Le Bras and Van Doren, 2006; Streit et al., 2002), as it is expressed at the tip of the testis, within hub cells, CySCs and GSCs, but undetectable in ovaries (Figure 1D, J–J''' and Figure S2) (Gönczy et al., 1992; Kiger et al., 2000; Streit et al., 2002). Characterization of the *shof* mutation revealed an 18kb insertion approximately 5kb downstream of the *esg* transcriptional start site (Figure S1F), and testes from flies carrying strong, loss-of-function *esg* alleles in combination with the *shof* mutation exhibited phenotypes similar to *shof* homozygotes, with loss of both GSC and CySC populations (Figure S1B–E), indicating that *shof* is an allele of *esg*. Accordingly, RNA *in situ* hybridization revealed a lack of *esg* expression in testes from newly eclosed *esg<sup>shof</sup>* males (Figure 1H). Furthermore, while *esg* expression was highly enriched at the anterior end of ~50% (53/93) of control embryonic gonads, *esg* RNA was absent from ~90% (61/70) of *esg<sup>shof</sup>* mutant gonads (Figure 2C–D), indicating that the *esg<sup>shof</sup>* mutation results in loss of *esg* expression at the testis tip from late embryogenesis and into adulthood.

### ***esg* is required for maintenance of apical hub cells**

The premature loss of early germline and somatic cells in testes from *esg<sup>shof</sup>* flies was accompanied by a reduction in hub cells and loss of function of the testis stem cell niche. Hub specification and formation appeared normal during embryonic stages 16 and 17 in *esg<sup>shof</sup>* embryos, based on hub cell morphology and marker expression (Figure 2A–F). Similar to *esg*, RNA *in situ* analysis revealed *upd* mRNA was expressed at the anterior tip of wild-type embryonic testes (Le Bras and Van Doren, 2006; Streit et al., 2002): roughly 50% of control embryonic gonads expressed *upd* (24/44, Figure 2A), as expected for a sexually dimorphic trait. However, contrary to the loss in *esg* expression (Figure 2C, D), approximately 50% of *esg<sup>shof</sup>* embryonic gonads (12/27) maintained *upd* expression (Figure 2AB). An additional hub marker, *center divider* (*cdi-lacZ*), was also detected in testes from *esg<sup>shof</sup>* animals during embryogenesis. Furthermore, early germ cells encircled the embryonic hub (Figure 2E, F), indicating that the hub was present and functional as an organizing center at this stage (Le Bras and Van Doren, 2006; Sheng et al., 2009; Wawersik et al., 2005).

Although hub formation and specification appeared unaffected in *esg<sup>shof</sup>* embryonic testes, disruption of normal hub morphology was detected during larval stages in mutant animals (Figure 2G–J). This first and most obvious defect in the *esg<sup>shof</sup>* mutant gonads preceded the progressive loss of GSCs and CySCs. Expression of Fas3, E-Cadherin (DE-Cad), and *cdi* (Le Bras and Van Doren, 2006) was markedly reduced or absent in hub cells from ~90% of 1-day old *esg<sup>shof</sup>* males (Figure S1A,B (n=18); Figure 3B,D). In addition, *upd* and *Drosophila* N-cadherin (DN-Cad) were lost, in most cases, by the second larval instar (L2) (Figure 2G, H and data not shown).

### ***escargot* is required autonomously for hub cell maintenance**

Loss of hub cells and stem cells in *esg<sup>shof</sup>* mutants could be due to cell intrinsic requirements for *esg* within all of the three cell types (hub, CySC, GSC). However, cell-type specific knock-down and rescue experiments suggested that *esg* is required in hub cells to maintain CySCs and GSCs, consistent with previous experiments suggesting that *esg* is required for

somatic cells to acquire or maintain hub cell fate (Voog et al., 2008). To probe the function of *esg* in hub cells, an *esg* RNAi construct was expressed specifically in hub cells using the bipartite GAL4-UAS expression system (Brand and Perrimon, 1993), in combination with the temperature sensitive *Gal80* allele (*Gal80<sup>ts</sup>*) (Lee and Luo, 1999). Use of *Gal80<sup>ts</sup>* permits tight control of gene expression during development and adulthood, as *Gal80<sup>ts</sup>* is active and inhibits GAL4 activity at 18°C but becomes inactive at 29°C. The *updGAL4* driver promotes expression of UAS constructs in hub cells but not in CySCs, cyst cells, GSCs, or their differentiating progeny. Flies carrying *updGAL4; UAS-esg<sup>RNAi</sup>/Gal80<sup>ts</sup>* (hereafter referred to as *esg<sup>RNAi</sup>*) were raised at 18°C to suppress transgene expression during development. Upon eclosion, flies were shifted to 29°C to induce *esg<sup>RNAi</sup>* expression within hub cells. Testes from *updGAL4, UAS-GFP;Gal80<sup>ts</sup>* (controls) and *esg<sup>RNAi</sup>* flies maintained at 18°C appeared similar to wild-type testes (data not shown). When shifted upon eclosion to 29°C and maintained there for 10 days, controls looked normal by phase contrast microscopy (Figure 3E) and expressed markers for hub cells, as well as early somatic and germ cells (Figure 3F). In contrast, testes from *esg<sup>RNAi</sup>* flies shifted to 29°C for 10 days strongly resembled those from 1-day old *esg<sup>shof</sup>* homozygotes displaying progressive loss of hub cells and expression of early somatic and germline markers (Figure 3C–D, G–H). In *esg<sup>RNAi</sup>* flies, only a few hub cells were detected, with an average hub cell number of 0.2 per testis (n=38) after 10 days at 29°C (Table S1). Control flies did not exhibit loss of hub cells (Table S1), as flies that did not carry the RNAi transgene maintained an average of 8.1 hub cells over 10 days (n=30). Taken together, these experiments suggest that *esg* is required autonomously within hub cells of adults to maintain hub integrity.

### ***escargot* is required autonomously in CySCs, but dispensable for GSC maintenance**

The function of *esg* was also required cell autonomously in CySCs for maintenance of somatic stem cells, as determined by generating positively marked (GFP<sup>+</sup>) CySC clones homozygous mutant for either of two strong, hypomorphic *esg* alleles by FRT-mediated recombination. One day post clone induction, 53% of control testes contained at least one marked CySC adjacent to the hub (n=54 testes), and 60% (*esg<sup>G66</sup>*, n=43) or 26% (*esg<sup>L2</sup>*, n=38) of testes examined contained at least one GFP<sup>+</sup> *esg* mutant CySC (Figure S4 A–E). Marked control CySC clones were observed in 39% (n=66), 33% (n=85), and 30% (n=61) of testes examined at 5, 10, and 15 days after clone induction, indicating that control CySCs are maintained over time (Figure S4 A–B, E). In contrast, CySCs homozygous mutant for *esg<sup>G66</sup>* (16%, n=43) or *esg<sup>L2</sup>* (5%, n=40) were quickly lost by 5 days after clone induction, and no *esg* mutant CySCs were observed after 10 (*esg<sup>G66</sup>*, n= 16) (*esg<sup>L2</sup>*, n=36) or 15 days (*esg<sup>G66</sup>*, n= 27) (*esg<sup>L2</sup>*, n=52) (Figure S4C–E). Mutant cyst cells expressed somatic differentiation markers, encapsulated germline cysts and no increase in CySC apoptosis was detected, indicating that *esg* is not absolutely required for cyst cell differentiation or survival but is required for CySC maintenance (Figure S4C and data not shown).

In contrast to a direct role for *esg* in hub cells and CySCs, no effect on GSC maintenance was observed when germline clones were generated that were mutant for either the *esg<sup>G66</sup>* or *esg<sup>L2</sup>* allele (Table S2), which is likely due to functional redundancy provided by the founding family member *snail* (G. Hime, unpublished data). These data confirm that the requirement of *esg* for GSC maintenance, as suggested by the *shof* phenotype, is non-cell

autonomous (Streit et al., 2002) and likely due to its autonomous role within the hub and/or CySCs.

### Expression of *escargot* in hub cells rescues hub loss in testes from *shof* flies

Expression of *esg* in hub cells was sufficient to rescue the *esg<sup>shof</sup>* phenotype. A GFP-tagged *Esg* construct (UAS-*esg<sup>NLAP</sup>*) was expressed in hub cells or cyst cells in the *esg<sup>shof</sup>* background (Figure 4A and Figure S5A–D), and rescue of the *esg<sup>shof</sup>* phenotype was observed when *esg<sup>NLAP</sup>* was expressed in hub cells (Figure 4B–F) but not in cyst cells (Figure S5F). The extent of rescue appeared to be dependent upon the level of *esg* expression, as shifting to higher temperatures to increase the activity of GAL4 resulted in an average hub cell number closer to wild-type (compare Figures 4G and S5E).

The carboxy-terminus of *Esg* contains five zinc finger domains, which are necessary for DNA binding and transcriptional regulation (Fuse et al., 1994). Expression of a truncated version of *esg* in which the C-terminal portion of the protein was deleted (UAS-*esg<sup>ZF</sup>*) (Figure 4A) was not sufficient to suppress loss of the hub and stem cells in *esg<sup>shof</sup>* males (Figure 4F), suggesting that the ability of *Esg* to regulate hub maintenance is dependent upon its ability to bind to DNA.

### Hub cells become cyst cells upon loss of *escargot*

No apoptosis was observed in hub cells during development in testes from *esg<sup>shof</sup>* males (Figure 2K, L and data not shown), raising the possibility that hub cells are lost due to a change in fate, rather than to cell death. Indeed, lineage-tracing experiments using the G-TRACE system, in combination with *esg<sup>RNAi</sup>* in hub cells suggested that hub cells become cyst cells upon loss of *esg*. The G-TRACE system provides a real-time readout of GAL4 activity (UAS-*dsRed* expression), as well as permanent labeling (*ubi-GFP*) of cells that are expressing GAL4 or were derived from GAL4 expressing cells (Evans et al., 2009) (Figure 5A–F, I, J). The G-TRACE cassette was utilized in combination with *Gal80<sup>ts</sup>* to suppress activation of RNAi and the lineage-tracing cassette during development.

Testes from control flies raised at 18°C and shifted to 29°C upon eclosion exhibited restricted expression of DsRed and GFP within hub cells, which co-stained with Fas3 (95.3%, n=64) (Figure 5A). However, three days after RNAi-mediated knockdown of *esg* within hub cells, GFP expression was detected in cells that appeared outside of the hub in the majority of testes examined (58.6%, n=70) (Figure 5B). Importantly, the increase in the total number of GFP<sup>+</sup> cells appearing outside the hub after 5 days was coincident with a loss of hub cells (Figure 5H; mean for control=8.5 hub cells compared to 6.8 for *esg<sup>RNAi</sup>*, p<0.01). After 10 days of RNAi induction, 100% (n=44) of testes from *esg<sup>RNAi</sup>* flies contained GFP<sup>+</sup> cells that were outside of the hub, in contrast to 1.7% (n=117) of control testes (Figure 5G). After 10 days, the average number of hub cells in *esg<sup>RNAi</sup>* flies was 1.8 hub cells/testis, compared to control flies, which was unchanged at 8.4 hub cells/testis (p<0.001, Figure 5H).

The GFP<sup>+</sup>/Fas3<sup>-</sup> cells in close proximity to the hub expressed high levels of Zfh-1 (Figure 5D, arrowheads) (Leatherman and Dinardo, 2008), whereas GFP<sup>+</sup> cells that were located

further away from the apical tip expressed the differentiation marker Eyes Absent (*Eya*) (Figure 5F, arrowheads), indicating that cells derived from the hub could differentiate along the cyst lineage. These data suggest that *Esg* is required to maintain hub cell identity by preventing conversion into somatic cyst cells. This is in contrast to other factors, such as Headcase, that appear to maintain hub cells by preventing loss due to programmed cell death (Resende et al., 2013).

Interestingly, analysis of hub cell fate using the G-TRACE system revealed that wild-type hub cells also convert to cyst cells during development (Figure 5I–K). When the system was suppressed prior to eclosion, 0% of testes showed GFP<sup>+</sup> cells outside of the hub [Figures 5K (n=18), 5G (n=23), and 6F (n=16)]. In contrast, when the system was active during development, this number increased significantly to 36% (n=50, Figure 5K). This is consistent with previous data demonstrating that hub cells and CySCs are derived from a common progenitor pool during development (Dinardo et al., 2011) and our observation that larval testes contain, on average, approximately twice as many hub cells as adults (Toledano et al., 2012a). Therefore, the conversion of hub cells to the cyst lineage may be a normal, programmed event during development of the male germ line.

### Cyst cells derived from hub cells can proliferate

Upon loss of *esg* activity in hub cells, the majority of GFP<sup>+</sup>/Fas3<sup>-</sup> cells expressed differentiation markers specific to cyst cells; however, the number of GFP<sup>+</sup> cells was greater than the overall number of hub cells, suggesting that hub cells which convert to the cyst lineage may have progressed through a mitotic CySC stage. Immunostaining for the mitotic marker phospho-histone H3 (pHH3) revealed that hub cell descendants were capable of cell division. GFP<sup>+</sup>/pHH3<sup>+</sup> mitotic, hub-derived cells were observed in 3/16 testes from *esg<sup>RNAi</sup>* flies (Figure 6A). In addition, after labelling *ex vivo* with the thymidine analogue, EdU, to mark cells progressing through S phase, at least one GFP<sup>+</sup>/EdU<sup>+</sup> hub cell was observed in 8/22 (36%) testes in which *esg<sup>RNAi</sup>* was expressed in hub cells. GFP<sup>+</sup>/EdU<sup>+</sup> cells were never observed in control flies (n=32) within the hub. Interestingly, we also found rare dsRed<sup>+</sup>/EdU<sup>+</sup> cells within hubs following *Esg* downregulation (Figure 6B–C), suggesting that some cells in the hub re-entered the cell cycle, consistent with loss of hub cell identity. Accordingly, downregulation in the expression of DEcad and DN-cad, two common markers of hub identity, was also observed after knockdown of *esg* (Figure S5G–J’).

While the overall number of GFP<sup>+</sup> cells increased approximately two-fold between 5 and 10 days (p<0.01), most hub cells were lost after 10 days (Figure 5H). Given the critical role that hub cells play in regulating CySC behaviour, this precluded our ability to determine if the converted hub cells were true CySCs or differentiated into cyst cells after one cell cycle. To assess the ability of converted hub cells to maintain CySC function, flies that expressed *esg<sup>RNAi</sup>* for 7 days (at 29°C) were shifted back to 18°C for 10 days to suppress transgene expression and further hub cell loss (Figure 6E, F). An approximate 7-fold increase in GFP<sup>+</sup> cells was observed over 10 days, when compared to flies that were maintained at 29°C (p<0.001, Figure 6D–F), indicating that a proportion of GFP<sup>+</sup>/Fas3<sup>-</sup> hub derived cells were capable of acting as *bona fide* CySCs, provided that active hub cells remained. Overall these

results suggest that *esg* maintains hub cell fate by blocking conversion to the cyst cell lineage

### Escargot interacts with the co-repressor CtBP, which is also required for maintenance of hub cell fate

In addition to expression in the testis, Esg is expressed in numerous other tissues and stem cell populations in *Drosophila*, including neural stem cells, known as neuroblasts, and the digestive tract (Ashraf et al., 1999b; Cai et al., 2001; Micchelli and Perrimon, 2006; Toledano et al., 2012b). To identify Esg interacting proteins that may be co-factors utilized to regulate cell fate decisions, we developed an unbiased biochemical purification strategy. The UAS-*esg*<sup>NLAP</sup> construct used to rescue the *esg*<sup>shof</sup> phenotype was adapted for use in cell culture such that stable S2 cell lines were generated that expressed Esg<sup>NLAP</sup> under an inducible promoter (Figure 7A) (Kyriakakis et al., 2008). Both one-step and two-step immuno-purification (IP) strategies were used to pull down the Esg-GFP fusion using anti-GFP antibodies, and protein interactors were identified by mass spectrometry (MS) (Table S3). Proteins that were also identified in control IPs were considered as background.

In both the one and two-step immunoprecipitation followed by mass spectrometry, the most abundant protein identified as judged by spectra counts was the co-repressor protein, C-terminal binding protein (CtBP) (Table S3). The interaction of Esg and CtBP was independently confirmed by repeating the immuno-precipitation and directly immunoblotting for CtBP (Figure 7B). Although Esg had never been shown to bind CtBP directly, it contains two CtBP binding domains (P-DLS-K), and other Snail family transcription factors are known to interact with CtBP (Ashraf et al., 1999a; Hemavathy et al., 2004; Nieto, 2002; Qi et al., 2008). Therefore, these experiments confirm a predicted interaction and demonstrate the validity of our approach.

CtBP is required cell autonomously for CySC maintenance (Leatherman and Dinardo, 2008), similar to Esg (Figure S4); however, CtBP also appears to play a cell autonomous role in maintaining hub cell fate. Immunofluorescence analysis revealed that CtBP is expressed in the nuclei of all cells at the apical tip of the *Drosophila* testis: hub cells, CySCs, and GSCs (Figure 7C). Expression of a *CtBP*<sup>RNAi</sup> construct specifically in hub cells of adults (genotype: *updGal4; UAS-CtBP*<sup>RNAi</sup>; *Gal80<sup>ts</sup>*) led to a loss of hub cells similar to that observed upon reduction of *esg* (Figure 7E, D; compare Figures 7E and 5H). While hub size was normal in adult flies that had been raised at 18°C, flies that had been shifted to 29°C to activate transgene expression displayed hub cell loss by 3 days. After 10 days of transgene expression, the mean number of hub cells per testis had decreased to 2.1 (n=28, p<0.001), with several testes displaying complete loss of the hub (Figure 7E).

Lineage-tracing experiments utilizing the G-TRACE system in combination with *CtBP*<sup>RNAi</sup> revealed that loss of CtBP in hub cells resulted in 93.9% of testes displaying GFP<sup>+</sup> cells outside of the hub after 10 days of RNAi induction (n= 49; Figure 7F). This was in contrast to control flies of the same genotype maintained at 18°C (6.1%, n=33), as well as control flies expressing the driver alone (genotype: *updGal4; Gal80<sup>ts</sup>*) (18°C; 5.9%, n=34) (29°C; 3.5%, n=142) (Figure 7F). Lastly, when *CtBP*<sup>RNAi</sup> flies were shifted back to 18°C to suppress transgene expression and further loss of hub cells, the total number of GFP<sup>+</sup> cells



increased, again suggesting a subset of hub-derived cells were maintained as CySCs (Fig 7F, I). The total number of GFP<sup>+</sup> cells at the 10 day time point was slightly higher in the *CtBP<sup>RNAi</sup>* flies than in *esg<sup>RNAi</sup>* flies, and remaining hub cell numbers were higher at all time points examined. *Zfh-1* has been shown to regulate the maintenance of CySCs in a CtBP-dependent manner (Leatherman and Dinardo, 2008). However, in contrast to loss of *Esg* or CtBP, targeted depletion of *Zfh-1* in hub cells did not result in loss of hub cells or conversion of hub cells into CySCs (Figure S6).

## Discussion

Given the integral role of the niche in regulating stem cell behaviour, changes in niche size or function could accompany alterations in stem cell activity during development or lead to decreased stem cell activity as a consequence of disease or aging (Jones and Wagers, 2008; Toledano et al., 2012a). Here we identify the Snail class transcriptional repressor *Escargot* as a factor that regulates the balance of cell fates within the stem cell niche. Our mapping and characterization of the *shutoff* mutation, in combination with targeted loss of function experiments, identified *Esg* as an essential factor that is required in hub cells to maintain niche integrity by blocking conversion of hub cells to the cyst cell lineage. Furthermore, immunoprecipitation followed by mass spectrometry identified the co-repressor protein, CtBP, as an interacting partner of *Esg* (Figure 7). Our data suggest that *Esg* acts in concert with CtBP in hub cells to regulate maintenance of hub cell identity. In addition to maintaining hub cell fate, *Esg* acts autonomously to regulate CySC maintenance and self-renewal. We predict that a systematic, functional analysis of other candidates identified through our IP/MS analysis (Table S3) will lead to the characterization of cell-type specific co-factors for *Esg*. These subsequent studies are likely to identify regulatory networks that provide insight into how *Esg* can act at nodes to regulate hub cell vs cyst cell identity, as well as stem cell behaviour in distinct tissues (e.g. testis vs intestine).

Although our data suggest that support cells within the testis stem cell niche (hub cells) can assume stem cell function, this is likely not a common event in adults. However, conversion of hub cells to CySCs may be a normal step of maturation of the testis niche during development (Figure 5J), which is restrained by *Esg* and its partners in adults. An alternative model is that hub cells are comprised of a pool of quiescent CySCs, although given the differences in gene expression observed between the two cell types, the transition between states would require more than cell cycle withdrawal.

Indeed, the close relationship between CySCs and hub cells is quite complex. Previous lineage-tracing and BrdU pulse-chase studies have suggested that CySCs can contribute to the pool of hub cells (Voog et al., 2008). For example, in experiments where 1 labelled CySC was generated, on average, using FLP-mediated genetic recombination, labelled hub cells were observed in 25.8% (49/190) and 24.5% (52/212) of testes examined at 5 and 10 days following clone induction, respectively. In contrast, another group used a similar FLP-mediated lineage-tracing strategy that resulted in 1.5 to 3.3 labelled CySCs per testis and did not observe labelled hub cells after 5 days (n=20) and observed labelled hub cells in 5% (1/20) of testes examined after 10 days (Dinardo et al., 2011). The reasons underlying the discrepancy between our findings and those described by Dinardo et al. remain unclear but

could be attributed to a number of factors, including genetic variation. Interestingly, we also reported that BrdU pulse-chase experiments in wild type animals resulted in labelled hub cells in 4% (n=143) and 3% (n=96) of testes examined at 5 and 10 days, respectively (Voog et al., 2008), frequencies that are closer to those reported by Dinardo et al. (Dinardo et al., 2011). In addition, subsequent experiments from our lab using the G-TRACE strategy as an alternative method for labelling all CySCs and their immediate progeny revealed an estimated rate of CySC contribution to the hub of 1.35% (see Materials and Methods for details). Given these data, we are less inclined to think of the CySC to hub cell transition as a homeostatic mechanism to maintain a specific number of hub cells and now favor a model where CySCs can become hub cells as a result of damage or a block in proliferative capacity. Indeed, recent findings from our lab suggest that contribution of CySCs to the hub may be affected by increased replicative stress in CySCs (Landais et al., 2014).

The intimate relationship between somatic stem cells and supporting niche cells observed in the *Drosophila* testis appears to be conserved in more complex systems. For example, mouse hair follicle stem cells can give rise to K6<sup>+</sup> niche cells (Hsu et al., 2011). In addition, in the mouse small intestine, Lgr5<sup>+</sup> crypt base columnar cells (CBCs) generate Paneth cells, which are a critical component of the stem cell niche at the base of each crypt (Sato et al., 2011). Interestingly, recent studies in the small intestine demonstrated that quiescent Paneth cell precursors could be stimulated to undergo significant proliferation upon injury, and thus recalled into an active stem cell state (Buczacki et al., 2013).

Although the hub is established normally during embryogenesis in *esg<sup>shof</sup>* males, *upd* expression is lost during development (Figure 2). Subsequent loss of hub cells and decreased Jak-STAT signalling is likely a major mechanism contributing to loss of tissue homeostasis. However, hub cells also express high levels of cell adhesion proteins, such as E-cadherin, N-cadherin, and Fasciclin. Therefore, one explanation for our findings could be that decreased expression of cell-cell adhesion molecules results in loss of hub cells upon loss of *esg* (Figure S5 H'-J'). However, several lines of evidence suggest that downregulation of conventional cell adhesion molecules is not the major mechanism underlying hub cell loss in *shof* mutants or upon RNAi-mediated depletion of *esg*: 1) RNAi-mediated depletion of *shotgun*, the gene encoding *Drosophila* E-cadherin (DEcad), in hub cells was not sufficient to induce hub cell loss (Michel et al., 2011; Voog et al., 2008), 2) loss of *esg* in individual hub cells did not result in downregulation of DE-cad (Voog et al., 2008) and 3) RNAi-mediated depletion of components of the exocyst complex, which traffics adhesion molecules to the cell's surface, in hub cells, resulted in disintegration of the hub, but not loss of hub cells (Michel et al., 2011). Attempts to overexpress cell adhesion molecules in hub cells to rescue the *shof* or *esgRNAi* phenotype resulted in lethality; therefore, we were unable to test this hypothesis directly. Data from chromatin profiling experiments, together with analysis of gene expression changes as a consequence of loss and gain of function of *Esg*, will provide specific transcriptional targets that could act downstream of *Esg* in hub cells and cyst cells to maintain cell identity and integrity of the testis niche.

Recent research has uncovered the remarkable ability of highly differentiated cells to become 'reprogrammed' into a stem cell-like state upon expression of a limited number of factors (Takahashi and Yamanaka, 2006). However, to date, the process of cellular

reprogramming has been studied primarily *in vitro* using cell cultures. Regeneration of tissues in vertebrates such as zebrafish, axolotl, salamanders, and mice suggest that de-differentiation may be possible *in vivo*, although it is not evident that differentiated cells in a blastema pass through a stem cell state (King and Newmark, 2012; Tanaka and Reddien, 2011). In contrast, spermatogonia can revert back to spermatogonial stem cells under certain conditions in the male germ line of both *Drosophila* and mice (Barroca et al., 2009; Brawley and Matunis, 2004; Kai and Spradling, 2004; Nakagawa et al., 2010). In addition, recent reports of de-differentiation of cells within the mammalian small intestine, stomach and lung into stem cells upon injury indicate that somatic tissues also possess this capacity (Buczacki et al., 2013; Stange et al., 2013; Tata et al., 2013), although the molecular mechanisms regulating de-differentiation are not well understood.

Our data reveal that differentiated niche support cells can also acquire stem cell properties upon removal of a single transcription factor *in vivo*, which underscores the importance of balancing cell fates within a niche and provides a tool to begin probing the mechanism by which this balance is achieved. Importantly, niche components may function more directly than previously appreciated to maintain an adequate number of stem cells available for tissue repair and regeneration. Furthermore, as data suggest that differentiated cells in the body naturally possess the ability to dedifferentiate *in vivo*, it may be possible to harness this activity to facilitate the repair of tissues without the need for cell transplantation. However, uncontrolled dedifferentiation could result in excess stem cells, which would disrupt tissue homeostasis and may even contribute to tumour initiation and growth. Therefore, characterization of genetic programs that maintain optimal niche function will provide a platform for designing niches to support the faithful derivation and maintenance of tissue stem cells in culture, facilitate the development of strategies to enhance the transplantation of stem cells in the course of regenerative medicine, and may provide novel targets for anti-cancer therapies.

## Materials and Methods

### Mapping the *esg<sup>shof</sup>* allele

The *shof* mutation spontaneously arose in *P571*, a stock carrying a *P{lacW}* insertion. The *P{lacW}* element in stock *P571* was recombined away from the *shof* locus. *shof* failed to complement *Df(2L)TE116(R)GW21* (Hiller et al., 2004) and *Df(2L)osp38* (BDSC #6082) but complemented *Df(2L)noc11* (BDSC #6080) and *Df(2L)TE116(R)GW2* (Hiller et al., 2004), placing *shof* near *esg*. Subsequent complementation tests revealed *shof/esg<sup>L2</sup>*, *shof/esg<sup>G66B</sup>* and *shof/esg<sup>P3</sup>* males had *shof* phenotypes.

Primers were designed using Primer 3 (<http://frodo.wi.mit.edu/>) to regions spanning 3584bp upstream and 6236bp downstream of *esg* start site. Qiagen Tissue Easy Kit was used to obtain genomic DNA from *OreR* and *S3-46*; *esg<sup>shof</sup>/esg<sup>shof</sup>* males. PCR fragments were sequenced by Eton Biosciences Inc., and analyzed using Chromas Pro. Sequences from *OreR* and *S3-46*; *esg<sup>shof</sup>/esg<sup>shof</sup>* were identical except for an ~18kb insertion in *S3-46*; *esg<sup>shof</sup>/esg<sup>shof</sup>* between 5228bp and 5840bp downstream of *esg* start site.

## Clonal analysis

For MARCM analysis adult flies were heat shocked two consecutive days at 37 °C for two hours and collected at indicated times for dissection and immunostaining (described below). Wild-type MARCM genotype: *y,w, hsflp<sup>122</sup>; FRT40A/FRT40A, tubGAL80; tubGAL4/2x-UAS-eGFP*. Mutant genotype: *esg<sup>G66</sup> (y,w, hsflp<sup>122</sup>; FRT40A esg<sup>G66</sup>/FRT40A tubGAL80; tubGAL4/2x-UAS-eGFP)*, and *esg<sup>L2</sup> (y,w, hsflp<sup>122</sup>; FRT40A esg<sup>L2</sup>/FRT40A tubGAL80; tubGAL4/2x-UAS-eGFP)*. Germline clonal analysis was carried out as described (Kiger et al., 2001a). Control genotypes used were: *y,w, hsflp<sup>122</sup>; FRT40A/FRT40A ubi-GFP*. Mutant genotypes used were: *esg<sup>G66</sup> (y,w, hsflp<sup>122</sup>; FRT40Aesg<sup>G66</sup>/FRT40A GFP)*, and *esg<sup>L2</sup> (y,w, hsflp<sup>122</sup>; FRT40Aesg<sup>L2</sup>/FRT40A GFP)*.

## Temporal expression of RNAi transgenes and G-TRACE cassette

All *updGAL4, UAS-GFP; UAS-esg-RNAi (esg-RNAi)* and *updGAL4, UAS-GFP; UAS-esg-RNAi/UAS-RedStinger, UAS-FLP, Ubi-p63 FRT>STOP FRT> nEGFP (esg-RNAi/G-TRACE)* flies were raised at 18°C unless noted otherwise. Control flies were kept at 18°C during adulthood while experimental flies were shifted to 29°C. This permitted visualization of cell fate changes in *esg<sup>-RNAi</sup>/G-TRACE* flies, as DsRed expression reflected hub cells under GAL4 control and descendants of DsRed<sup>+</sup> cells are permanently GFP<sup>+</sup> (Evans et al., 2009). To further regulate GAL4 activity, the *Gal80<sup>ts</sup>* transgene was used. Flies were raised at 18°C for 5 days post-eclosion to restrict Gal4 during final developmental stages. Flies were shifted to 29°C for 5–10 days to inhibit *Gal80<sup>ts</sup>* and activate Gal4. For pulse-chase experiments, flies were shifted back to 18°C to suppress GAL4 and track GFP<sup>+</sup> cells.

For G-TRACE experiments described in the Discussion, which measured the rate of CySC to hub cell conversion, *c587-Gal4; UAS-GFP, UAS-RedStinger, UAS-FLP, Ubi-p63 FRT>STOP FRT> nEGFP; tub-Gal80<sup>ts</sup> (c587-Gal4<sup>ts</sup>>G-TRACE)* flies were raised at 18°C. Three days after eclosion, the flies were shifted to 29°C for 2 days, then 25°C for 15 days. Control groups were kept at 25°C for 17 days (to control for spurious activation of G-TRACE), or 1 day at 29°C (to estimate baseline G-TRACE activation). Subtracting background control rates from the experimental rate showed an average of 1 additional hub cell in 20% of testis (n=48). Because this paradigm labeled all CySCs (as opposed to approximately one/testis in previous experiments), it is difficult to extrapolate a comparable rate of CySC to hub contribution. However, if we divide the observed rate by an average 15 CySCs per testis, then estimated contribution is 1.35%.

## Creation of NLAP stable cell lines and fly strains

The pMK33-NTAP (GS) vector (Kyriakakis et al., 2008) (gift from Alexey Veraksa), was modified with GFP in the IgG domain, resulting in the pMK33-NLAP (N-terminal Localization and Affinity Purification) vector. The coding region of *escargot* was cloned into the NLAP vector to generate the *Esg<sup>NLAP</sup>* fusion protein. pMK33-*Esg<sup>NLAP</sup>* and pMK33-NLAP were transfected into S2 cells according to the manufacturer's recommendations (Fugene HD, Roche). Seventy-two hours after transfection, cells were transferred into media containing 150ug/ml hygromycin (Sigma). Over 3 weeks a stable, hygromycin resistant population was selected.

To generate the UAS-NLAP and UAS-Esg<sup>NLAP</sup> flies, the pMK33-NLAP pMK33-Esg<sup>NLAP</sup> plasmids were sub-cloned into the pUAST-attB plasmid and injected into embryos at the att2 site on chromosome 3 by Genetic Services Inc. (Sudbury, MA).

### Immunoprecipitations

The expression of Esg<sup>NLAP</sup> and NLAP was induced adding 0.35mM copper sulfate. After 17–24 hours cells were lysed in TAP buffer (50mM Tris, pH7.5, 5% glycerol, 0.2% IPEGAL, 1.5mM MgCl<sub>2</sub>, 125mM NaCl, 25mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mMDTT, 1mM EDTA, Roche complete protease inhibitors) and the soluble extract incubated with rabbit anti-GFP (Clontech) for 3 hours. IgG dynabeads (Life Technologies) were added for 1.5 hours and samples were washed 3X in TAP buffer. Bound proteins were immunoblotted with either mouse anti-GFP (1:1000, Millipore) or anti-CtBP (1:2000, D. Arnosti). Chemiluminescent detection was performed according to the manufacturer's protocol (ECL plus, GE Healthcare).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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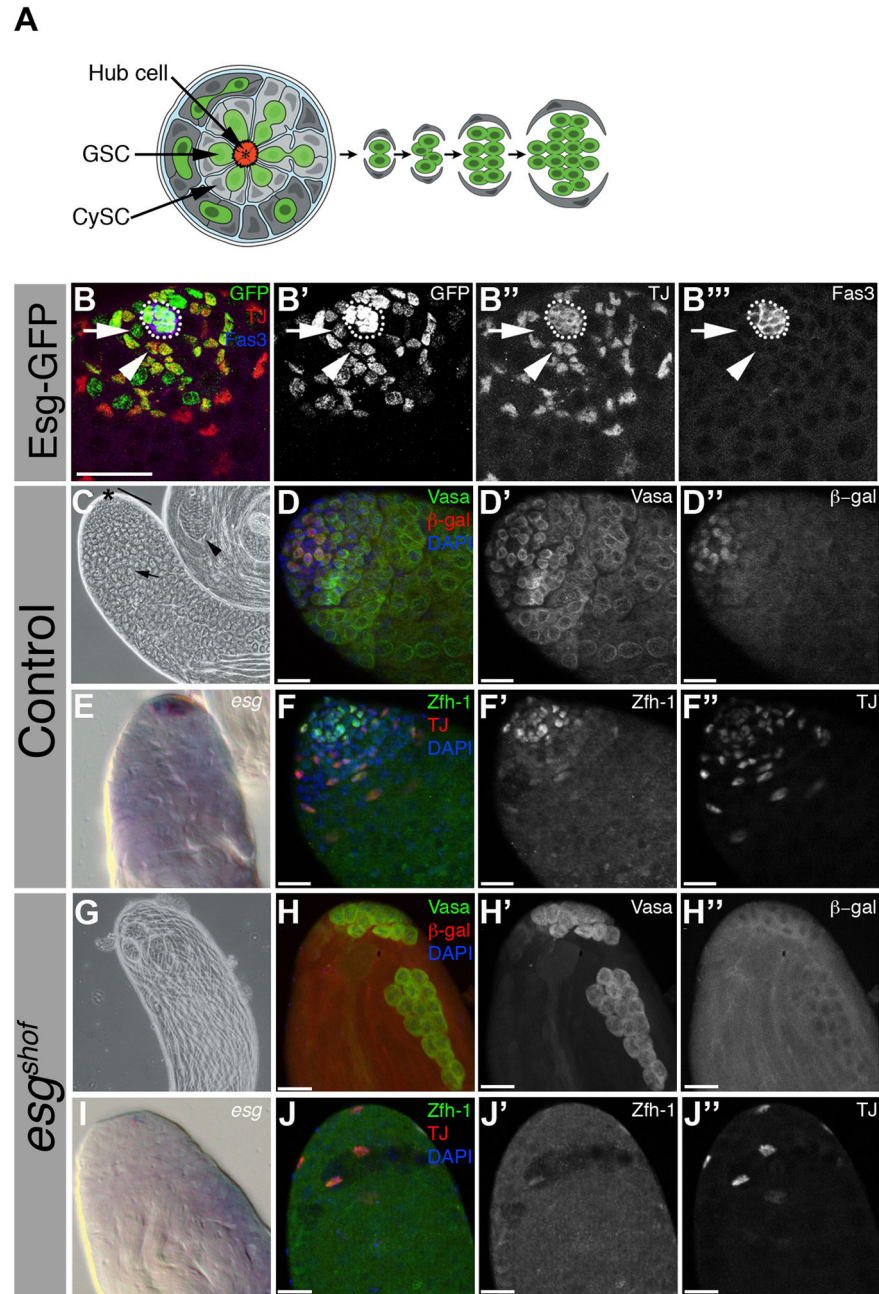
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### Highlights

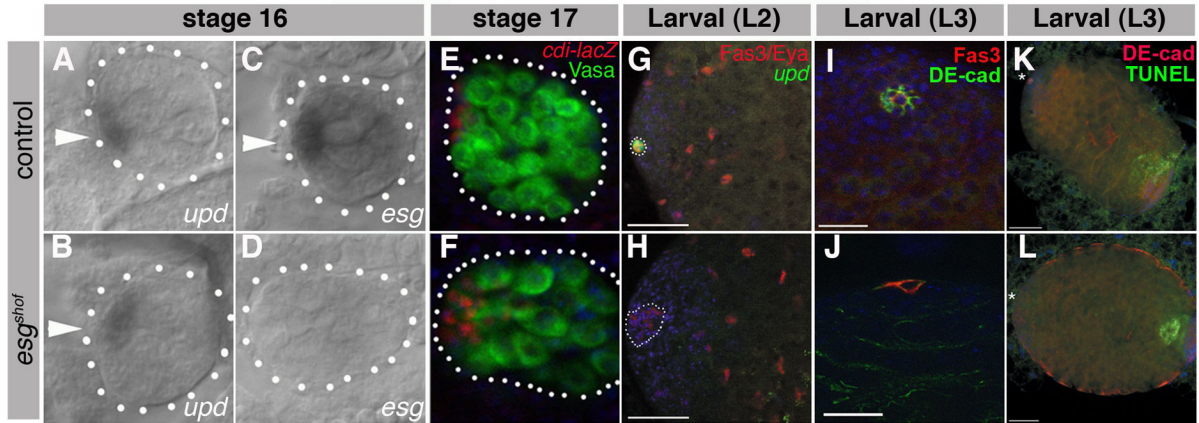
- Loss of Escargot (Esg) in hub cells results in conversion to the cyst lineage
- Hub cells depleted of Esg acquire properties of cyst stem cells (CySCs)
- Esg interacts with the co-repressor, C-terminal Binding Protein (CtBP)
- CtBP is also required for maintenance of hub cell fate
- Niche support cells can acquire stem cell traits



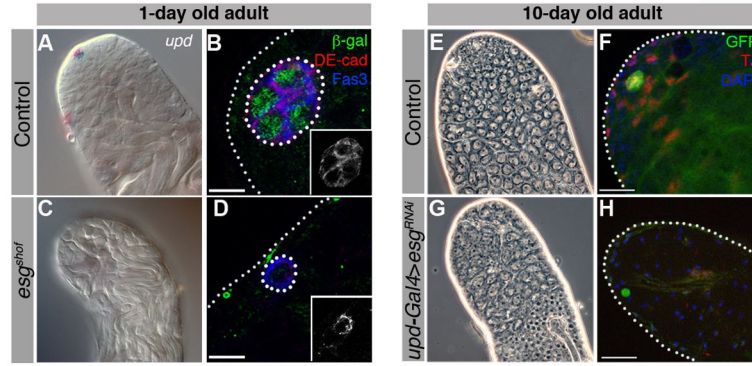
### Figure 1. The stem cell niche is lost in adult *esg<sup>shof</sup>* males

(A) Schematic cross-section of testis tip. Germline stem cells (GSC, light green) contact hub cells (red) and somatic cyst stem cells (CySC, light grey). GSCs divide asymmetrically to self-renew and produce goniatoblasts, which generate spermatogonial cysts (dark green) via transit amplifying (TA) divisions. CySCs generate cyst cells (dark grey) and encapulate spermatogonia. Phase contrast images of testes from (B) 1-day old control and (F) *esg<sup>shof</sup>* males. (B) Asterisk: apical tip. Bar: transit amplification zone. Arrow: spermatocytes. Arrowhead: spermatids. (F) Note spermatids in *esg<sup>shof</sup>* testis. Wild-type (C) and *esg<sup>shof</sup>* (G) flies with *S3-46-lacZ* enhancer trap immunostained for Vasa (green, C', G'),  $\beta$ -gal (red, C'', G''),

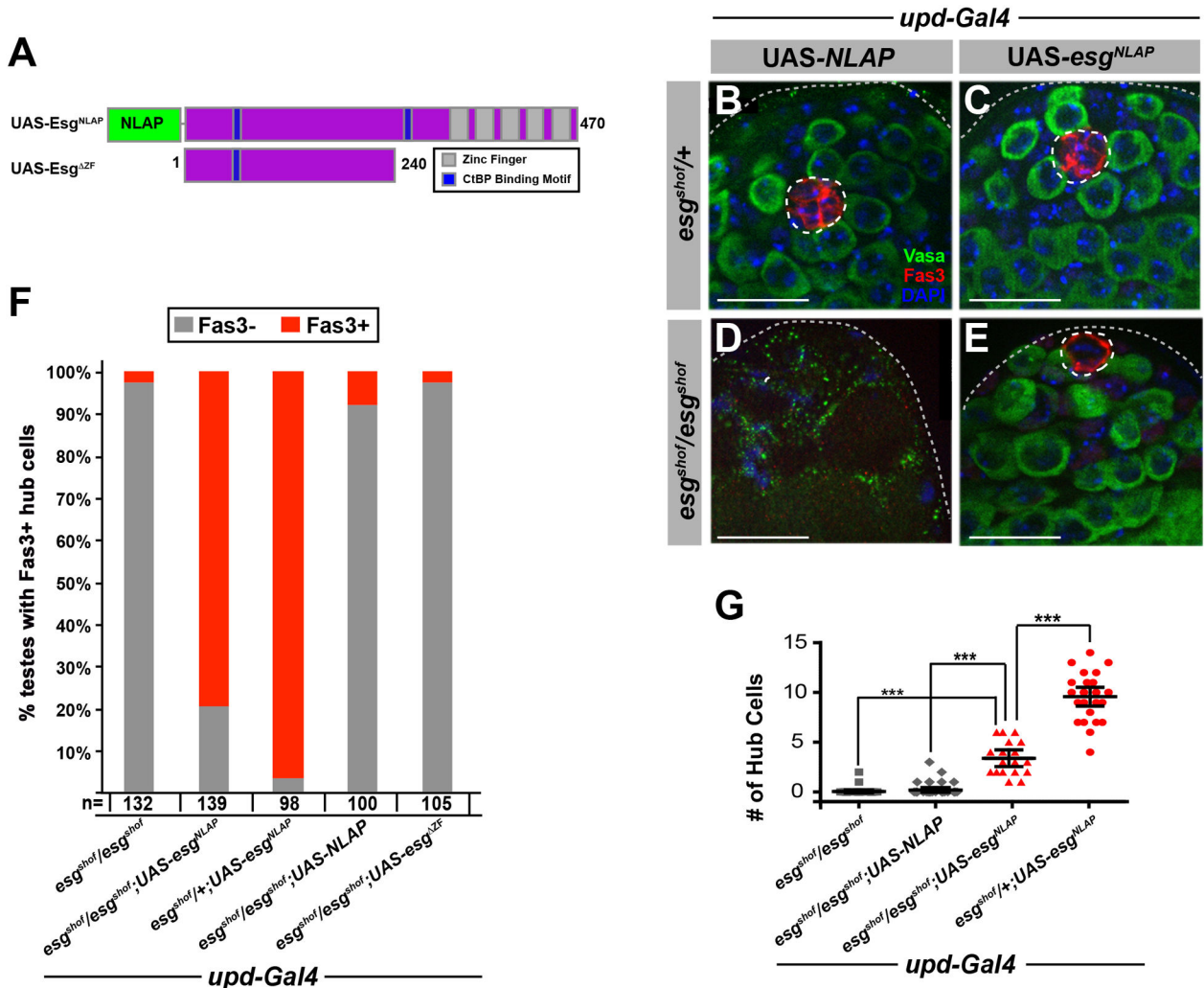
G<sup>''</sup>), DAPI (DNA, blue). (C) Vasa marks germ cells and *S3-46* marks GSCs and early spermatogonia. (G) *esg<sup>shof</sup>* testes lack *S3-46* expression and contain late stage Vasa+ germ cells. (E, I) Testes immunostained for early somatic cell markers Zfh-1 (green, E', I') and Traffic Jam (TJ, red, E'', I'') in control testes (E), and largely absent in *esg<sup>shof</sup>* (I). (D, H) DIC images of RNA *in situ* for *esg* in control (D) and *esg<sup>shof</sup>* (H) adult testes. (J) Adult testis in *esg*-GFP enhancer trap testes immunostained for GFP (green, J'), TJ (red, J''), and Fasciclin 3 (Fas3, blue, J'''). Hub cells (outline), GSCs (arrow), and CySCs (arrowhead) express GFP. Scale bars, 20 μM (C, E, G, I) and 10 μM (J).



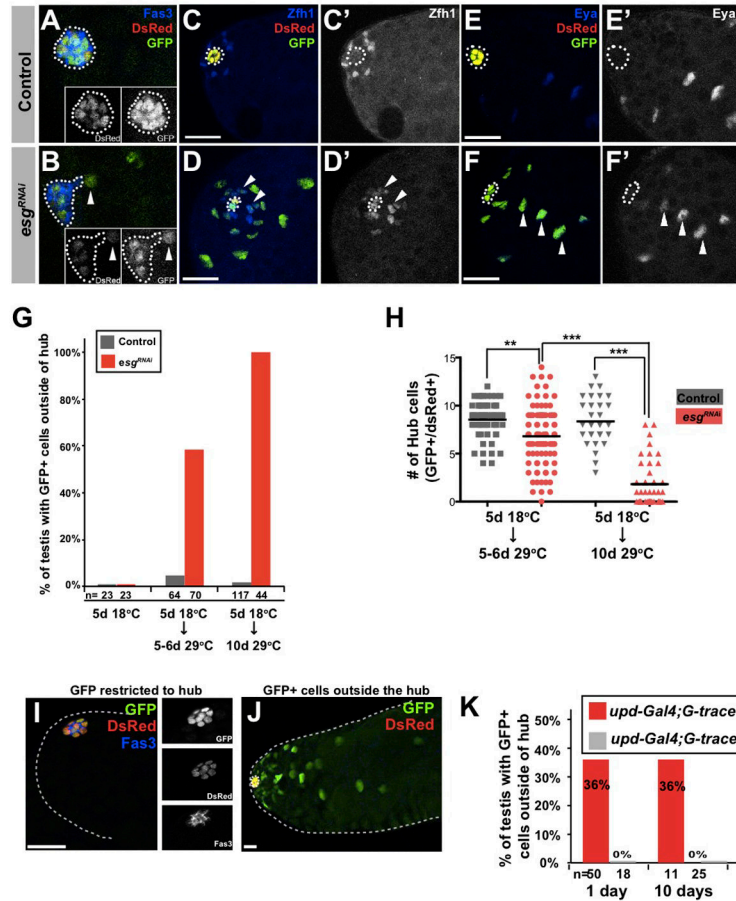
**Figure 2. Loss of hub marker expression during larval development in *esg<sup>shof</sup>* males**  
 DIC images of RNA *in situ* for *upd* (A,B) or *esg* (C,D) in control (A, C) and *esg<sup>shof</sup>* (B, D) stage 16 embryonic gonads (outlined, arrowheads). Gonads from *cdi-lacZ* (E) and *cdi-lacZ*; *esg<sup>shof</sup>* (F) stage 17 embryos (outlined) stained for Vasa (green),  $\beta$ -galactosidase (red), DAPI (blue). Larval L2 *updGAL4*, UAS-*GFP* (G) and *updGAL4*, UAS-*GFP*; *esg<sup>shof</sup>* (H) gonads stained for Fas3 (red, outline), Eyes Absent (Eya, red), GFP (green), DAPI (blue). Note loss of GFP expression in (H), despite residual Fas3. Larval L3 control (I) and *esg<sup>shof</sup>* (J) gonads stained for Fas3 (red), E-cad (green), DAPI (blue). Larval L3 *updGal4*, UAS-*GFP* (K) and *updGal4*, UAS-*GFP*; *esg<sup>shof</sup>* (L) gonads stained for E-cadherin (red, asterisk), DAPI (blue), and TUNEL assay for apoptotic cells (green).



**Figure 3. Esg is required autonomously in hub cells to maintain the stem cell niche** (A, C) DIC images of RNA *in situ* for *upd* in 1-day old wild-type (A) or *esg<sup>shof</sup>* (C) flies. (B, D) Testes from 1-day old *cdi-lacZ* (B) or *esg<sup>shof</sup>; cdi-lacZ* (D) flies immunostained for Fas3 (blue), DE-cadherin (DE-Cad, red, insets),  $\beta$ -gal (green). Phase contrast image of testes from 10-day old *updGal4, UAS-GFP; Gal80<sup>ts</sup>* (E) or *updGal4; UAS-esg<sup>RNAi</sup>/Gal80<sup>ts</sup>* (G) flies shifted to 29°C upon eclosion to induce transgene expression. Immunofluorescence images of testes from 10-day old *updGal4, UAS-GFP* (F) and *updGal4, UAS-GFP; UAS-esg<sup>RNAi</sup>* (H) males for GFP (green, hub), TJ (red) and DAPI shifted to 29°C upon eclosion. Scale bars, 10  $\mu$ M.



**Figure 4. The *esg<sup>shof</sup>* phenotype is rescued by hub specific expression of Esg**  
**(A)** Schematic of Esg protein with N-terminal GFP LAP tag. Compare *esg* construct without zinc fingers (UAS-*esg<sup>ZF</sup>*) (Fuse et al., 1994). Two CtBP binding domains are depicted (aa #40–46, PQDLCVK and aa#259–265, PEDLSLK) **(B–E)** UAS-*esg<sup>NLAP</sup>* **(E)**, but not control transgene (UAS-*NLAP*) **(D)** expression under *updGAL4* control resulted in rescued hub in *esg<sup>shof</sup>* flies. **(C)** Overexpression of UAS-*esg<sup>NLAP</sup>* had no effect on hub in *esg<sup>shof</sup>* heterozygotes. **(B–E)** Testes from 3–5 day-old flies immunostained for Vasa (germline, green), Fas3 (hub, red), DAPI (blue). Scale bars, 20 $\mu$ M. **(F)**. Proportion of testes from 3–5 day old adult flies with Fas3<sup>+</sup> hub cells in given genotype. **(G)** Hub cell number quantification in genotypes from panels **(C–E)** and *esg<sup>shof</sup>* homozygous (DAPI used to count individual cells). UAS-*esg<sup>NLAP</sup>* expression rescues hub loss, although the total number of hub cells was less than in the *esg<sup>shof</sup>* heterozygote ( $p < 0.001$ ).



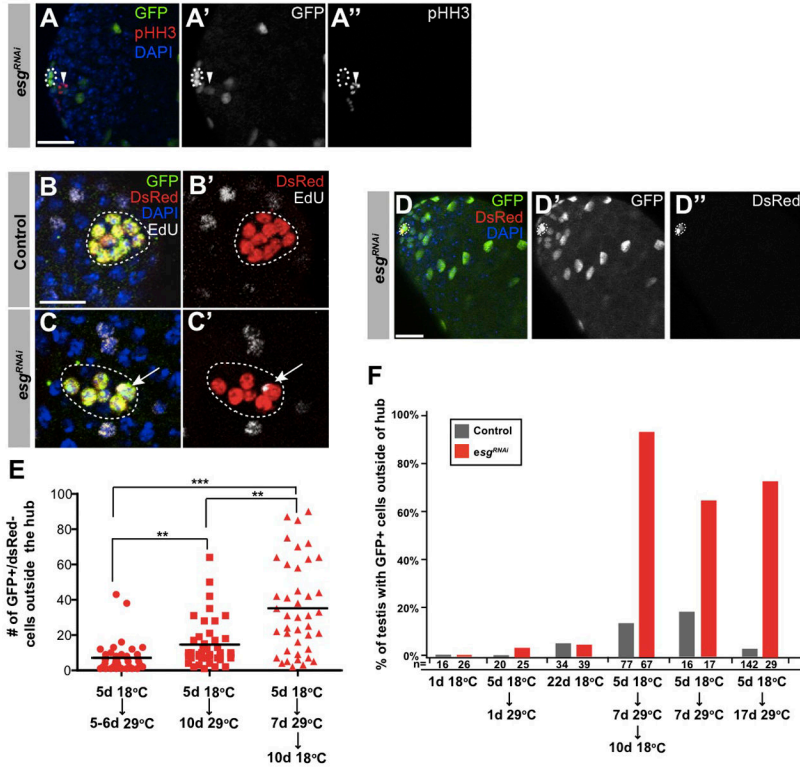
### Figure 5. Loss of *esg* results in hub cell-cyst cell conversion

(A–F) Immunofluorescence images of testes in control (*updGal4*, *UAS-GFP*; *G-TRACE*) (A,C,E) and *updGal4*, *UAS-GFP*; *G-TRACE*; *UAS-esg<sup>RNAi</sup>* (B,D,F) flies raised at 18°C and shifted to 29°C for 5 days to induce transgene expression. Testes immunostained for GFP (green), DsRed (red), Fas3 (blue, outline, A–B), Zfh-1 (blue, arrowheads, C–D'), Eyes absent (*Eya*, blue, arrowheads, E–F'). Hub outlined in all panels. Scale bars, 20μM.

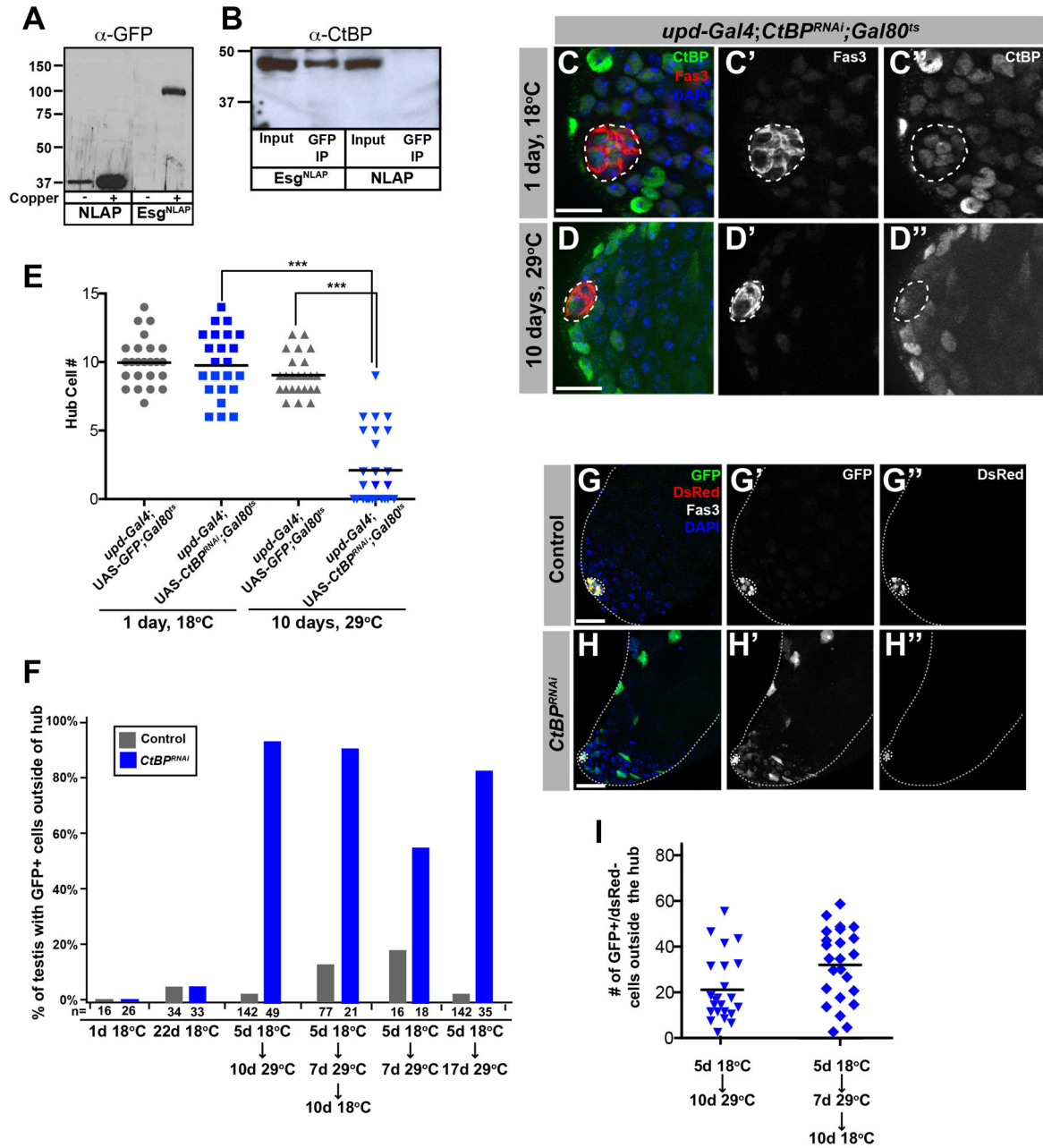
Frequency of hub-cyst cell conversion (G) and quantification of GFP<sup>+</sup>/DsRed<sup>+</sup> hub cells (H) in control (*updGal4*; *G-TRACE*; *Gal80<sup>ts</sup>*) and *esg<sup>RNAi</sup>* (*updGal4*; *G-TRACE*; *UAS-esg<sup>RNAi</sup>*/*Gal80<sup>ts</sup>*) flies raised and maintained at 18°C for 5 days after eclosion, then shifted to 29°C for 5 or 10 days. The mean number of GFP<sup>+</sup>/DsRed<sup>+</sup> cells in testes from *esg<sup>RNAi</sup>* flies was significantly lower after 5 days at 29°C (6.8, \*\**p*<0.01), which decreased further after 10 days (*esg<sup>RNAi</sup>*, 1.8; control, 8.4, \*\*\**p*<0.001). Statistical significance shown with one-way ANOVA (Kruskal-Wallis test) and Dunn's multiple comparison test. (I–J) *updGal4*; *G-TRACE* flies with restricted hub GFP expression pattern (I), GFP<sup>+</sup> cells outside of the hub (J). Testes immunostained for GFP (green, top inset) and Fas3 (blue, bottom inset). DsRed expression detectable without immunostaining (red, middle inset). Scale bars, 20μM. (K) Quantification of testes containing GFP<sup>+</sup> cells outside the hub in *updGal4*; *G-TRACE*; *Gal80<sup>ts</sup>* flies raised at 18°C and shifted to 25°C upon

eclosion (OFF during development) or *updGal4*; *G-TRACE* flies raised and maintained at 25°C for 10 days (ON during development).





**Figure 6. Hub cells that lack Esg convert to functional CySCs**  
**(A)** Testes from *updGal4*, *UAS-GFP*; *G-TRACE*; *UAS-esg<sup>RNAi</sup>* flies immunostained for GFP (green), phospho-histone H3 (pHH3, red), DAPI (blue). Scale bars, 20µM. **(B–C)** Immunofluorescence images from control (*updGal4*; *G-TRACE*; *Gal80<sup>ts</sup>*) **(B–B')** and *updGal4*; *G-TRACE*; *UAS-esg<sup>RNAi</sup>/Gal80<sup>ts</sup>* **(C–C')** flies raised and maintained at 18°C for 5 days after eclosion, then shifted to 29°C for 5 days. Testes immunostained for GFP (green), DsRed (red), EdU (white) to mark cells in S-phase. Scale bars, 10µM. **(D–D'')** *updGal4*; *G-TRACE*; *UAS-esg<sup>RNAi</sup>/Gal80<sup>ts</sup>* flies shifted to 18°C for 10 days after 7 days at 29°C and immunostained for GFP (green), DsRed (red), DAPI (blue). Scale bar, 20µM **(E)** Quantification of the number of GFP<sup>+</sup>/DsRed<sup>-</sup> cells. Statistical significance shown with one-way ANOVA (Kruskal-Wallis test) and Dunn’s multiple comparison test (\*\*p<0.01, \*\*\*p<0.001). **(F)** Quantification of GFP<sup>+</sup> cells outside of the hub for noted experimental paradigms. After 17 days at 29°C, ~ 25% of *esg<sup>RNAi</sup>* testes lacked GFP<sup>+</sup> cells likely reflecting complete hub loss.



**Figure 7. CtBP binds to Esg and is required for hub maintenance**

(A) Western blot analysis from stable line extracts confirmed expression of Esg<sup>NLAP</sup> and NLAP with copper induction. Expected sizes of NLAP (33.3kDa) and Esg<sup>NLAP</sup> (85.3kDa). (B) Immunoprecipitation with anti-GFP antibodies and Western blot with anti-CtBP antibodies confirmed Esg and CtBP interaction. Expected size of CtBP (42.3 kDa). (C,D) Testes from 1-day old adult *updGal4;UAS-CtBP<sup>RNAi</sup>;Gal80<sup>ts</sup>* flies raised at 18°C (C) or shifted to 29°C for 10 days (D) immunostained for CtBP (green) and Fas3 (hub, red). Scale bars, 10  $\mu$ M. (E) Quantification of hub cell number in genotypes. Statistical significance shown with one-way ANOVA (Kruskal-Wallis test) and Dunn's multiple comparison test (\*\*\* $p < 0.001$ ). (F) Frequency of hub cell-cyst cell conversion in control (*updGal4; G-*

*TRACE/Gal80<sup>ts</sup>* or *CtBP<sup>RNAi</sup>* (*updGal4; UAS-CtBP<sup>RNAi</sup>; G-TRACE/Gal80<sup>ts</sup>*) flies. Testes with no detectable GFP<sup>+</sup> cells at the 17 day-29°C time point were scored as negative. **(G–H)** Immunofluorescence images from control **(G)** or UAS-*CtBP<sup>RNAi</sup>* **(H)** flies raised and maintained at 18°C until 5 days after eclosion, shifted to 29°C for 7 days, and shifted back to 18°C. Testes immunostained for GFP (green), DsRed (red), and Fas3 (white). **(I)** Quantification of GFP<sup>+</sup>/dsRed<sup>-</sup> cells for the noted temperature regimes.