Supplemental Methods:

Fly husbandry and stocks

Flies were raised at 25°C on standard cornmeal-molasses media. Fly stocks were from the Bloomington *Drosophila* Stock Center (BDSC), Vienna Drosophila Stock Center (VDRC), or generous gifts as indicated: esg^{P3} (esg^{05729}) and esg^{L2} (S. Hayashi) (Hayashi et al., 1993); esg^{G66B} (Whiteley et al., 1992) and *S3-46lacZ* (S. DiNardo); *esg-GFP*^{P01986} (http://flytrap.med.yale.edu/); UAS-esg^{RNAi} (VDRC #1437), UAS-$CtBP^{RNAi}$ (VDRC #107313), *E132Gal4* (*upd*Gal4) (Halder et al., 1995) and *c-587Gal4* (T. Xie); *upd*Gal4,UAS-*GFP* (E. Bach); *nanosGAL4:VP16* (M. Van Doren) (Van Doren et al., 1998), G-TRACE (UAS-*RedStinger*, UAS-*FLP, Ubi-p63* FRT>STOP FRT> *nEGFP)* (U. Banerjee) (Evans et al., 2009); esg^{VS2} (esg^{35Ce-1}), *Df(2L)Exel8034* (*esg* deficiency chromosome, BDSC #7830), cdi^{07013} (BDSC #11711), $Gal80^{ts}$ (BDSC #7018). Wild-type flies were *Oregon R.*

Immunostaining and microscopy

Testes were dissected, fixed, and immunostained as described previously (Voog et al., 2008) or were fixed in 4% PFA in PLP buffer (0.075M lysine, 0.01M sodium phosphate buffer pH7.4) for 30min and washed in PBS with 0.1% Triton X-100. RNA in-situ hybridization (Boyle et al., 2007), and ex-vivo EdU incorporation (Wong and Jones, 2012) were performed as described previously.

Antibodies used were: rabbit anti-Vasa (1:2000, P.Lasko), rabbit anti-Zfh-1 (1:5000, R. Lehmann), guinea pig anti-Zfh-1 (1:3000, C. Doe), rabbit anti-CtBP (1:1000, D. Arnosti), rabbit anti-b-galactosidase (1:2000, Cappel), guinea-pig anti-TJ (1:3000, D. Godt), mouse anti-phospho histone H3 (Cell Signaling, 1:500), either rabbit anti-GFP (1:5000, Molecular Probes) or mouse anti-GFP (1:200), and anti-DsRed (1:100, Clontech). Mouse anti-Fasciclin 3 (7G10, 1:50), mouse anti-Eyes Absent (1:10), rat anti-DE-cadherin (DCAD2, 1:20), and rat anti-DN-cadherin (DN-Ex #8, 1:20) were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. Secondary antibodies were diluted 1:500 (Life Technologies). Samples

were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Samples were analysed with a Zeiss Axiovert 200 microscope and processed using AxioVision (version 4.8; C. Zeiss) and Adobe Photoshop. Analysis of testes with G-TRACE cassette were performed using either a **Zeiss LSM 710/780 Laser Scanning confocal microscope (Figures 4G-J, 5, and 6)** or a Leica TCS SP2 AOBS confocal microscope and LCS Lite 2.61.1537 software (Leica Microsystems) **(Figure 4A-F)**. Phase contrast and DIC images were obtained using a Leica DM5000 microscope equipped with a DC500 camera using Firecam imaging software (version 1.7.1; Leica Microsystems). GraphPad Prism was used for all statistics.

Mass Spectrometry

Immunoprecipitations were conducted with 3-5x10^9 cells, which were induced for 3 hours with 0.35mM copper sulfate. After binding to IgG dynabeads, samples were washed 3X in TAP buffer and then equilibrated into TEV cleavage buffer (10mM 1M Tris, pH7.5, 100mM NaCl, 0.1% IPEGAL, 0.5mM EDTA, 1mM DTT). 60 units of AcTEV protease (Invitogen) was added to the beads overnight at 4°C. Eluates were collected and added to streptavidin beads (Invitrogen) and rotated at 4°C for 2 hours. Beads were then washed 3X in TEV cleavage buffer and eluted with 2mM Biotin in 10mM Tris, pH 7.5. For mass spectrometry, TEV cleavage eluates (1-step IP) or streptavidin binding eluates (2-step IP) were collected, denatured in 8M urea, and reduced and alkylated with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (Roche) and 55 mM iodoacetamide (Sigma-Aldrich) respectively. The samples were digested over-night with trypsin (Promega) according to the manufacturer's specifications.

The protein digests were pressure-loaded onto 250 micron i.d. fused silica capillary (Polymicro Technologies) columns with Kasil frit packed with 3cm of 5 micron Partisphere strong cation exchange (SCX) resin (Whatman) and 3 cm of 5 micron C18 resin (Phenomenex). After desalting, each bi-phasic column was connected to a 100 micron i.d. fused silica capillary (Polymicro Technologies) analytical column with a 5 micron pulled-tip, packed with 10 cm of 5 micron C18

resin (Phenomenex). Each MudPIT column was placed in line with an 1100 quaternary HPLC pump (Agilent Technologies) and the eluted peptides were electrosprayed directly into an LTQ mass spectrometer (Thermo Scientific). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B) and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). A six-step MudPIT was run with salt pulses of 0%, 20%, 40%, 70% and 100% buffer C and 90% buffer C/10% buffer B. The 120 minute elution gradient had the following profile: 15% buffer B beginning at 10 minutes to 55% buffer B at 90 minutes to 100% buffer B from 98 minutes to 108 minutes. A cycle consisted of one full scan mass spectrum (300-2000 m/z) followed by five data-dependent collision induced dissociation (CID) MS/MS spectra. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by Xcalibur data system (Thermo Scientific).

MS/MS spectra were extracted using RawXtract (version 1.9.9) (McDonald et al., 2004). MS/MS spectra were searched with the Sequest algorithm (Eng et al., 1994) against a FlyBase database concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). The Sequest search was performed using no enzyme specificity and static modification of cysteine due to carboxyamidomethylation (57.02146). Sequest search results were assembled and filtered using the DTASelect (version 2.0) algorithm (Tabb et al., 2002), requiring peptides to be at least half tryptic and a minimum of two peptides per protein identification. The protein identification false positive rate was below 2%. Of proteins identified in the NLAP sample (118), 86 and 79 were identified in the Esg-NLAP 1-step and 2-step IP samples respectively. These proteins were removed from the Esg interacting proteins list (Supplemental Table 3).

Supplemental References:

Eng, J.K., McCormack, A.L., and Yates, J.R. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. Journal of the American Society for Mass Spectrometry *5*, 976-989.

Evans, C.J., Olson, J.M., Ngo, K.T., Kim, E., Lee, N.E., Kuoy, E., Patananan, A.N., Sitz, D., Tran, P., Do, M.T*., et al.* (2009). G-TRACE: rapid Gal4-based cell lineage analysis in Drosophila. Nat Methods *6*, 603-605.

Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. Science (New York, N.Y *267*, 1788-1792.

Hayashi, S., Hirose, S., Metcalfe, T., and Shirras, A.D. (1993). Control of imaginal cell development by the escargot gene of Drosophila. Development *118*, 105-115.

McDonald, W.H., Tabb, D.L., Sadygov, R.G., MacCoss, M.J., Venable, J., Graumann, J., Johnson, J.R., Cociorva, D., and Yates, J.R., 3rd (2004). MS1, MS2, and SQT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. Rapid communications in mass spectrometry : RCM *18*, 2162-2168.

Peng, J., Elias, J.E., Thoreen, C.C., Licklider, L.J., and Gygi, S.P. (2003). Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. Journal of proteome research *2*, 43-50.

Tabb, D.L., McDonald, W.H., and Yates, J.R., 3rd (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. Journal of proteome research *1*, 21-26.

Van Doren, M., Williamson, A.L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Curr Biol *8*, 243-246.

Whiteley, M., Noguchi, P.D., Sensabaugh, S.M., Odenwald, W.F., and Kassis, J.A. (1992). The Drosophila gene escargot encodes a zinc finger motif found in snail-related genes. Mech Dev *36*, 117-127.

Supplemental Figures:

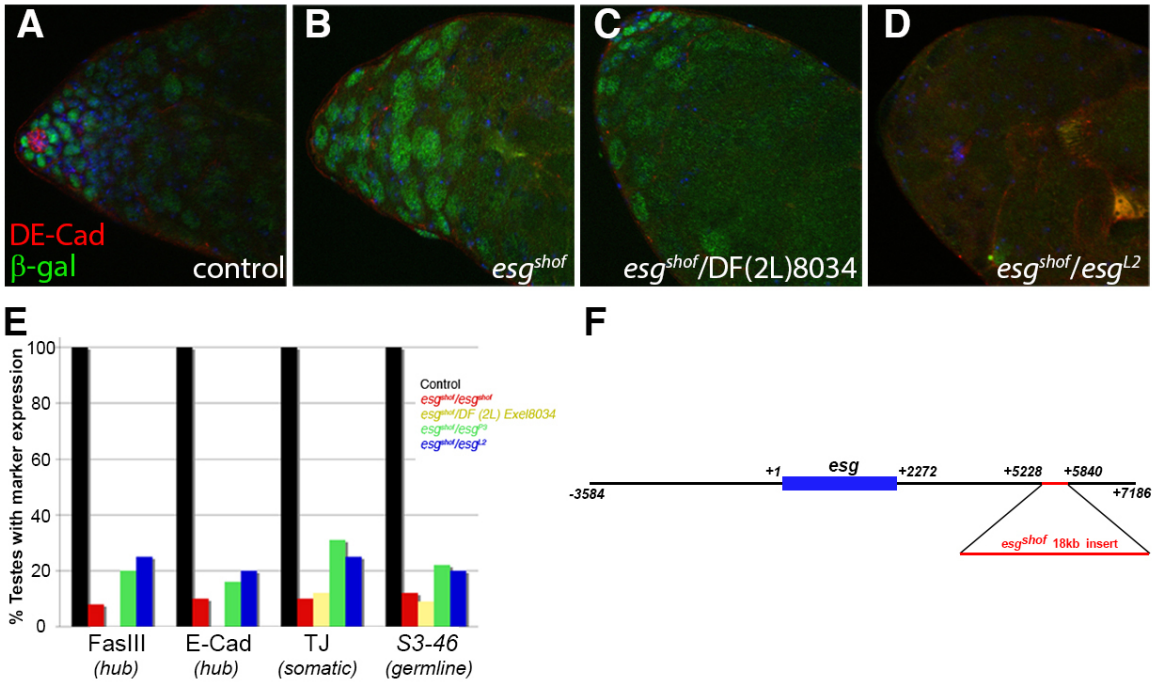

Figure S1: *shutoff (shof)* is an allele of *escargot.* (**A-D**) Testes from 1-day old adults stained for *Drosophila* E-Cadherin (red) and β-galactosidase (green), expressed from the *S3-46 lacZ* enhancer trap line. (**A**) Control (*S3-46*), (**B**) *S3-46*; esg^{shof}, (**C**) *S3-46*; esg^{shof}*/Df(2L)Exel8034,* (**D**) *S3-46*; esg^{shof}/esg^{L2}. (**E**) Hub, early somatic and early germline marker expression in testes from flies of the designated genotypes. (**F**) The esg^{shof} mutation is caused by an 18 kb insertion approximately 5.2kb downstream of the *esg* transcriptional start site. Complements data in Figure 1.

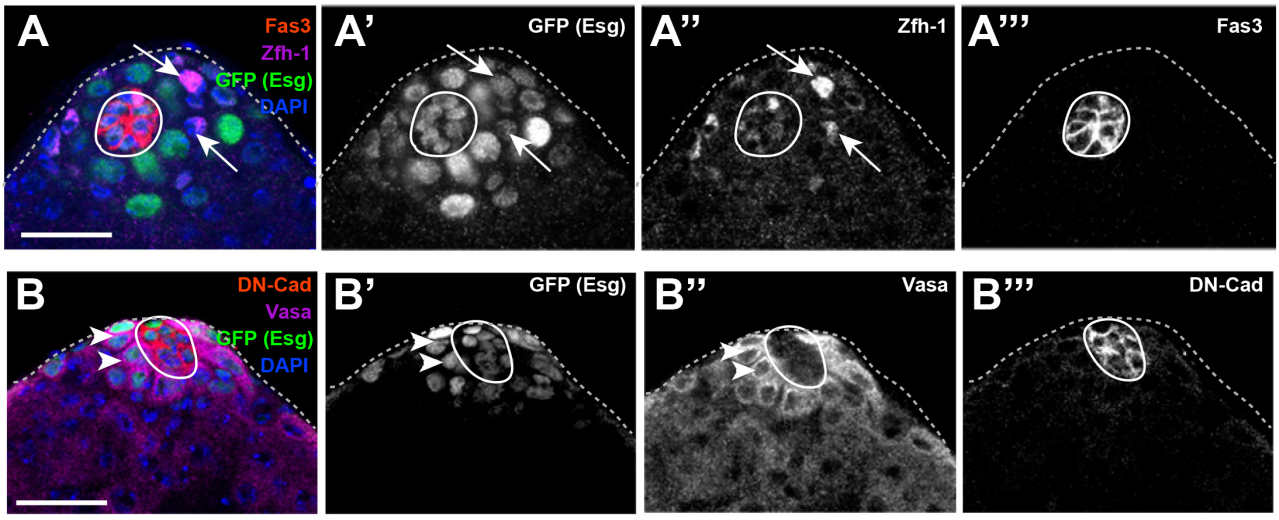

Figure S2: Esg is expressed in hub cells, cyst stem cells, and germline stem cells. (**A**) Immunofluorescence images of an adult testis from a fly carrying an *esg-GFP* enhancer trap immunostained for GFP (green, A'), Zfh-1 (magenta, A''), and Fasciclin 3 (Fas3, hub, outline, red, A'''). Arrows depict CySCs that are positive for both Esg and Zfh-1. (**B)** Testis from an *esg-GFP* fly immunostained for GFP (green, B'), Vasa (magenta, B''), and DN-cadherin (hub, outline, red, B'''). Arrowheads depict GSCs that are positive for both Esg and Vasa. Complements data in Figure 1.

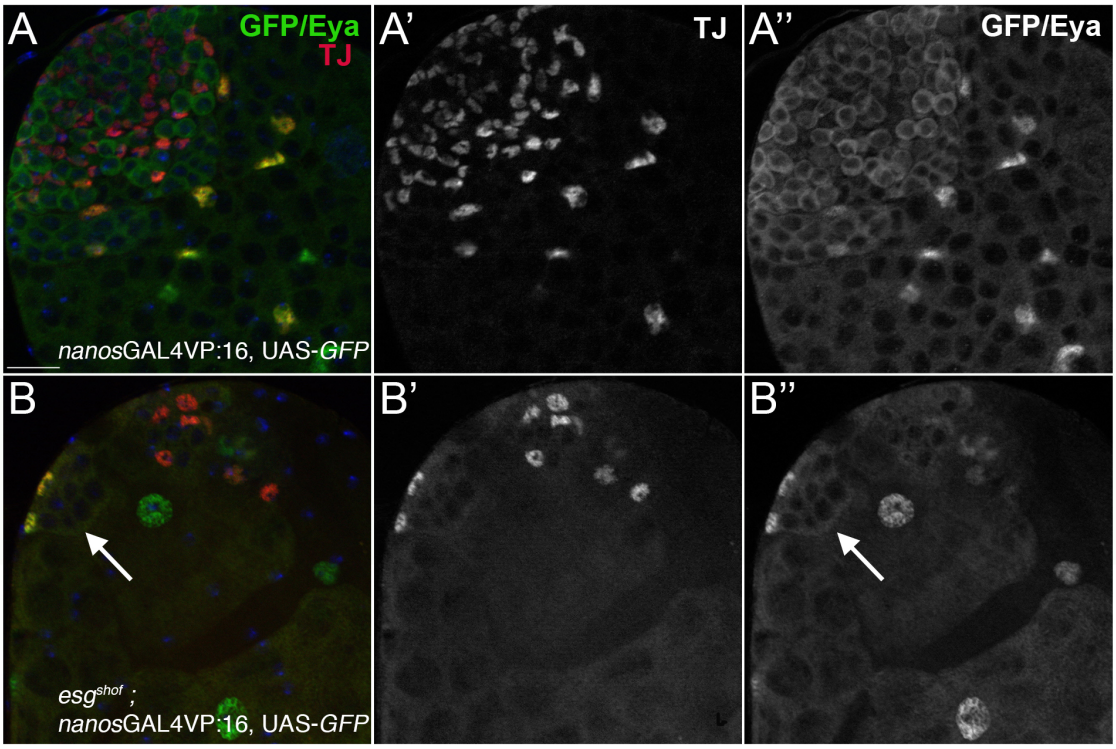

Figure S3. Testis from *esg*^{shof} males lack both germline and cyst stem cells. (**A-B**) Immunofluorescence images of adult testes from (**A**) *nanos-Gal4:VP16*, UAS-*tub:GFP* and (**B**) *esg*^{shof}*; nanos-Gal4:VP16*, UAS-*tub:GFP* flies immunostained for TJ (red), Eya (green), and GFP (green). Arrow in B denotes 8-cell spermatogonial cyst at the apical tip. Complements data in Figure 1.

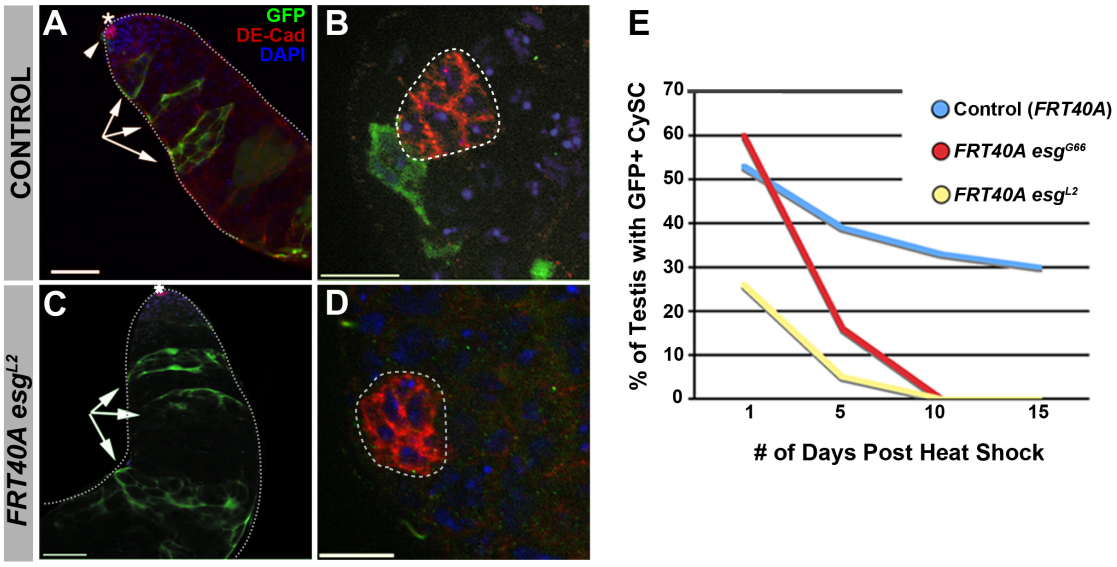

Figure S4: Esg is required autonomously for maintenance of CySCs. (**A**) Immunofluorescence image of progeny (arrows, green) from a marked CySC (arrowhead) adjacent to the hub (asterisk) 5 days after clone induction. (**B**) High magnification view of (**A**) showing a single, marked CySC (green) in contact with the E-cad^{+} (red) hub. (**C**) *esg*^{L2} mutant CySC clones are not maintained near the hub (E-cad^{+}, red) 5 days after clone induction. (**D**) CySC mutant *esg*^{L2} clones are absent at the tip 10 days after clone induction. (**E**) Percentage of testes with GFP^{+} CySC clones at 1, 5, 10, and 15 days after clone induction. Complements data in Figure 3.

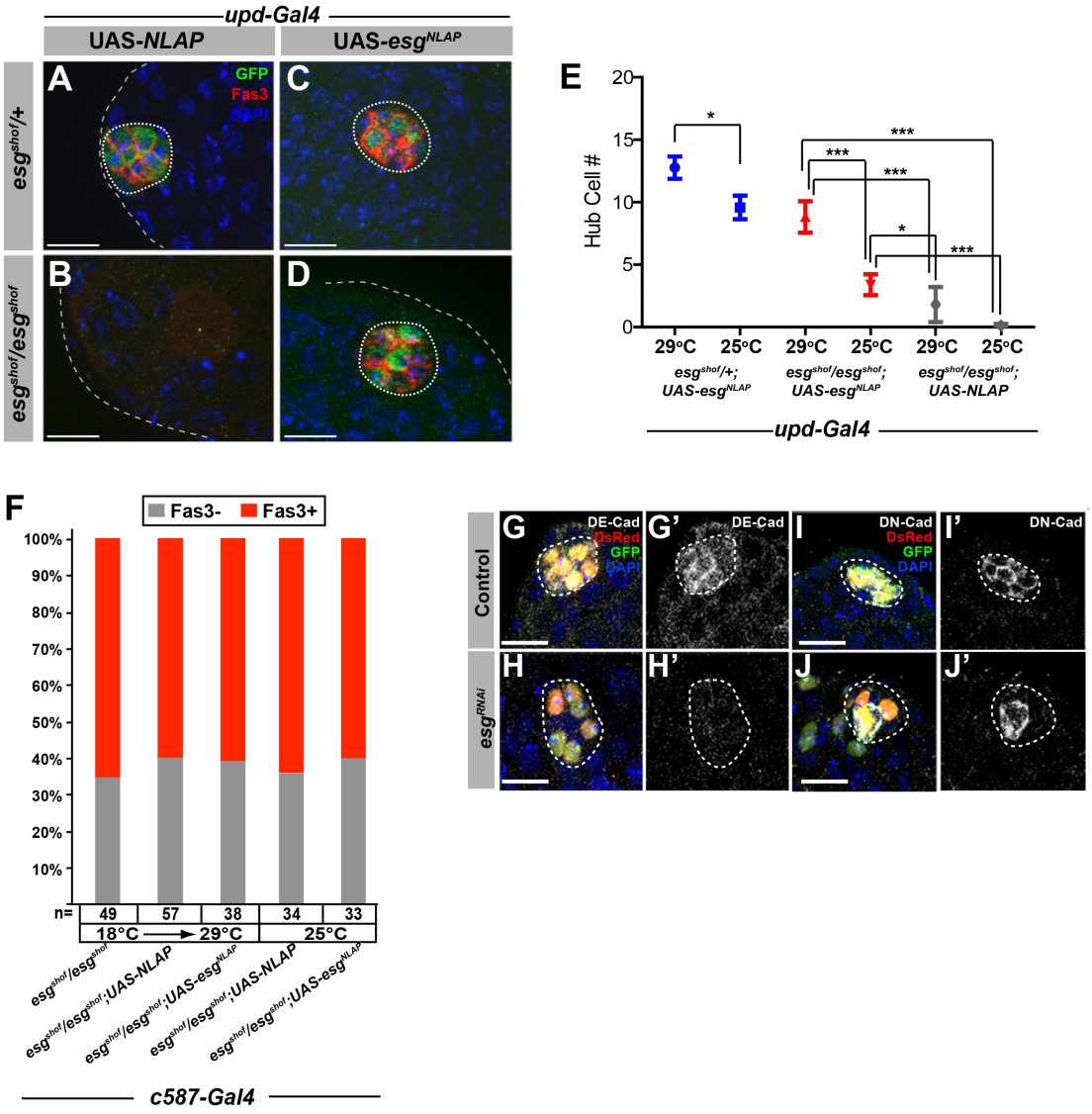

Figure S5. Expression of *esg* in the hub rescues loss of the hub in testes from *esg*^{shof} males. Testes from 1-3 day old adult *updGal4*; *esg*^{shof} /+;UAS-NLAP (**A**), *updGal4*; *esg*^{shof}/*esg*^{shof};UAS-NLAP (**B**), *updGal4*; *esg*^{shof} /+; UAS-*esg*^{NLAP} (**C**) and *updGal4*; *esg*^{shof}/*esg*^{shof}; UAS-*esg*^{NLAP} (**D**) flies were immunostained for GFP (green, recognizes NLAP tag) and Fas3 (red). Scale bars, 10μM. (**E**) Quantification of hub cell number in testes from 3-5 day old adult flies of the genotypes described in (**B**), (**C**) and (**D**) raised at either 25°C or 29°C. (**F**) Quantification of hubs (Fas3^{+} cells) in testes from 1-3 day old adult *c587-Gal4;esg*^{shof}/*esg*^{shof}, *c587-Gal4;esg*^{shof}/*esg*^{shof};UAS-NLAP, or *c587-*

Gal4;esg^{shof}/esg^{shof};UAS-*esg^{NLAP}* flies raised at 18°C and shifted to 29°C for 4-6 days before eclosion or maintained at 25°C throughout development. (**G-J**) Immunofluorescence images of testes from control (*updGal4;G-TRACE;Gal80^{ts}*) (**G**,**I**) and *updGal4;G-TRACE;UAS-esg^{RNAi}/Gal80^{ts}* (**H**,**J**) flies raised and maintained at 18°C until 5 days after eclosion, then shifted to 29°C for 5 days. Testes were immunostained for GFP (green) and DsRed (red), as well as DE-Cadherin (white, **G-H'**) or DN-Cadherin (white, **I-J'**). Hubs outlined in all panels. Scale bars, 10μM. Complements Figure 4.

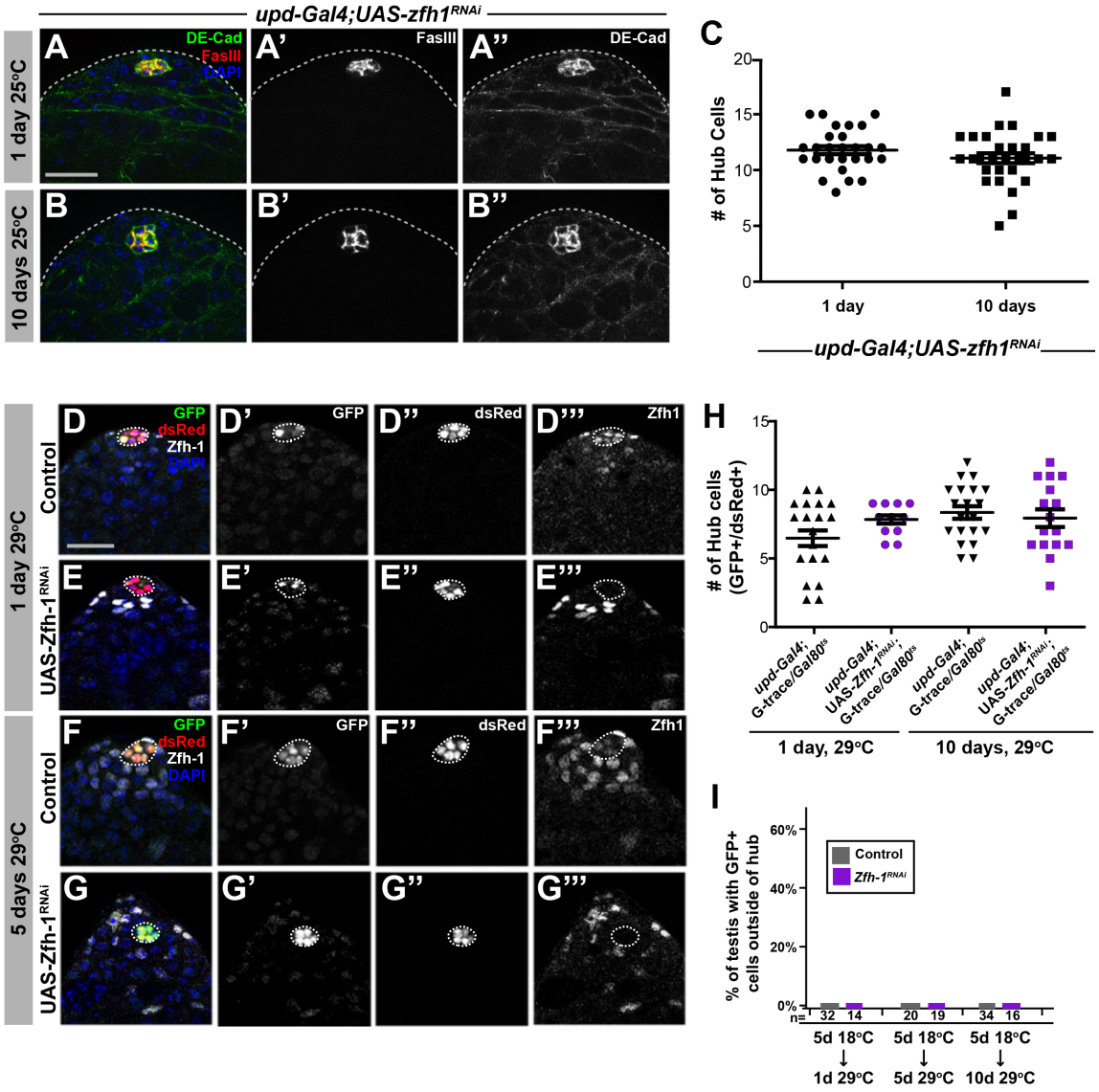

Figure S6. Zfh-1 is not required to prevent hub cells from converting to CySCs. (**A**) Testes from 1-day old (**A**) and 10-day old (**B**) adult *upd-Gal4*;UAS-*Zfh-1*^{RNAi} flies that were raised and maintained at 25°C were stained for Fas3 (red, **A', B'**), DE-Cadherin (green, **A'', B''**). Hub cells (scored as being both Fas3 and DE-Cad positive) were quantified from both 1 and 10 day old flies. Average hub cell number was not significantly different between the two conditions when compared using a Mann-Whitney U test. (**D-I**) *updGal4*;;*GTRACE*/*Gal80*^{ts} (Control) or *updGal4*; UAS-*Zfh-1*^{RNAi}; *GTRACE*/*Gal80*^{ts} flies were raised at 18°C and then moved to 29°C for either 1 (**D-E**), 5 (**F-G**), or 10 days (immunostain not

shown, but data used in **H-I**). Testes were dissected and immunostained for GFP (green, **D'-G'**), dsRed (red, **D''-G''**), Zfh-1 (white, **D'''-G'''**) and DAPI (blue). Testis were also stained for DE-Cadherin (data not shown) to verify that GFP+/dsRed+ cells were still expressing hub markers after 10 days in control and UAS-*Zfh-1*^{RNAi} flies. (**H)** Quantification of GFP^{+}/dsRed^{-} cells for the indicated genotype at each time point. No statistically significant differences were observed between each group when compared using a one-way ANOVA (Kruskal-Wallis test) followed by a Dunn's multiple comparison test. (**I**) For each genotype and timepoint, testes containing GFP+, dsRed- cells outside of the hub were quantified. Scale bars, 20μM. Complements Figures 5-7.