INVESTIGATING THE ROLE OF THE HISTONE DEMETHYLASE *LID* IN THE *DROSOPHILA* TESTIS NICHE

by
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ABSTRACT

Histone modification enzymes are highly expressed in the *Drosophila melanogaster* male germline stem cell niche. One of these modifiers, little imaginal discs (Lid), is a histone demethylase that specifically targets the H3K4me3 mark. Whereas studies have shown that activation of the Janus kinase signal transducer and activator of transcription (JAK-STAT) signaling pathway in cyst stem cells is crucial for proliferation and maintenance of germline stem cells (GSCs), the function of Stat92E in germ cells is believed to only regulate GSC-hub adhesion through DE-Cadherin. Here we show that Lid functions to regulate *Stat92E*, the downstream transcription factor for the JAK-STAT signaling pathway, in *Drosophila* male germ cells. Our results reveal that Lid is a positive regulator of *Stat92E* in germ cells through a derepression mechanism: Lid represses *Ptp61F*'s inhibitive effect on *Stat92E*. Loss of *lid* in germ cells results in a decrease in the number of GSCs. Our data suggest that activation of the JAK-STAT signaling pathway in germ cells is required for proper GSC proliferation and maintenance. Therefore, our study provides new insights into the functions of the JAK-STAT signaling pathway in the *Drosophila* male GSC niche, specifically in germ cells, as well as helps to better understand the biological role of histone demethylase *in vivo*.

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INTRODUCTION

Adult stem cells have the remarkable ability to undergo asymmetric mitotic divisions that produce two distinct daughter cells. One daughter cell maintains the stem cell properties and regenerative potency while the other differentiates to replenish specialized cell types. Stem cells reside in microenvironments, or niches, that provide signals from surrounding cells to the stem cells to prevent differentiation. The *Drosophila melanogaster* male germline stem cell (GSC) niche is one of the best characterized stem cell niches in which GSCs associate with two types of somatic cells: hub cells and cyst stem cells (CySCs). The GSCs, each cradled between two CySCs, form a rosette around the hub. The GSCs normally undergo asymmetric division radially away from the hub; the daughter cell that maintains contact with the hub remains the stem cell whereas the other daughter cell becomes a gonialblast. The gonialblast then undergoes four rounds of mitosis, developing from a two-cell spermatogonial cyst to a sixteen-cell spermatogonial cyst, before becoming spermatocytes, the precursor to mature sperm. Defects in the preservation of GSC identity lead to GSCs that overproliferate without differentiation, which may result in cancer, whereas premature differentiation of GSCs leads to an inability to maintain continuous sperm production.

The associations between GSCs, hub cells, and CySCs play major roles in the maintenance of GSCs. In addition, epigenetic regulation, such as histone modifications, is essential for maintaining stem cell identity and function. Researchers have shown that methylation of H3K4, K36 or K79 is generally found at genes that are activated whereas H3K9, K27 and H4K20 methylation is more commonly found at genes that are repressed (Li et al., 2010). Trithorax group (TrxG) proteins are crucial ‘epigenetic writers’ that are

responsible for trimethylating lysine 4 of the histone 3 tail (H3K4me3) (Schuettengruber et al., 2011). Countering the effect of these TrxG proteins is a histone demethylase called Little Imaginal Discs (Lid), named after the phenotype seen in *lid* mutant larvae (Gildea et al., 2000); *lid* mutant flies have significantly smaller imaginal discs during larval stages compared to wild type (wt). Lid shares all of the domains of the human JARID1 family of proteins: a JmjN domain whose function is unknown, a JmjC domain that is crucial for demethylase activity, an ARID (A/T rich interaction domain (Kortschak et al., 2000)) required for binding both A/T and G/C rich DNA sequences (Tu et al., 2008 and Scibetta et al., 2007), a C_5HC_2 zinc finger, and three PHD fingers (plant homeobox domain (Aasland et al., 1995)) involved in histone tail binding as well as mediating protein-protein interactions (Taverna et al., 2007). Lid is also the only *Drosophila* JARID1 family protein (Blair et al., 2011) and past studies have shown that knockdown of *lid* in *Drosophila* cells leads to an increase in the global levels of H3K4me3 (Lee et al., 2007 and Eissenberg et al., 2007). However, Lid's role in the maintenance of the testis niche is still not well understood. Thus, using various genetic and biochemistry methods, we investigated the function of Lid in the *Drosophila* male GSC niche.

When analyzing *lid* whole fly mutants, we found that the loss of Lid causes a decrease in the number of GSCs. Using cell-type specific drivers to knockdown *lid* in either germ cells or somatic cells, we found that Lid function was required only in germ cells in order to maintain a wt number of GSCs. When spectrosomes and branched fusomes were labeled in *lid* mutant flies, four-cell cysts were seen directly associated with the hub cells. In addition, *Bam* (*bag-of-marbles*, a gene required to initiate male gametogenesis) was ectopically expressed in cells next to the hub where the GSCs are

supposed to be. These data pointed towards the premature differentiation of GSCs as an explanation for the decreased number of GSCs in the *lid* mutant. Lastly, antibody staining along with qRT-PCR showed that the level of Stat92E in *lid* mutant testes is significantly lower than that of wt testes. Because the JAK-STAT pathway is known to be one of the main signaling pathways to play a critical role in the proper maintenance of the GSC niche (Tarayrah et al., 2013), we believe that this loss of JAK-STAT signaling in *lid* mutant testes could result in the loss of GSCs.

MATERIALS AND METHODS

Drosophila Stocks

Flies were raised on standard yeast/molasses medium at 25°C. The following stocks were used: lid^{10424} (Bloomington Stock Center, BL-12367), lid^{k06801}(Bloomington Stock Center, BL-10403), w^{1118}; *Df(2L) BSC184* (Bloomington Stock Center, BL-9612), lid^{k06801},*FRT40A* (Bloomington Stock Center, BL-111088), *UAS-lid shmiRNA* (Valium 10, TRiP.HM05155 from Bloomington Stock Center, BL-28944), *UAS-lid shmiRNA* (Valium 22, TRiP.GL00612 from Bloomington Stock Center, BL-36652), *upd-Gal4* (from D. Harrison, University of Kentucky, Lexington, KY, USA), *nanos-Gal4* (from M. Van Doren, Johns Hopkins University, Baltimore, MD, USA), *c587-Gal4* (from A. Spradling, Carnegie Institution Department of Embryology, Baltimore, MD, USA), *y,w; Ubi-GFP, Ubi-GFP, FRT40A* (Bloomington Stock Center, BL-5189), $hs\text{-}FLP^{122}$ (Bloomington Stock Center, BL- 33216), *UAS-lid*, $UAS\text{-}lid^{JmjC}$, $UAS\text{-}lid^{JmjN}$, $UAS\text{-}lid^{PHD1}$, $UAS\text{-}lid^{PHD3}$, $UAS\text{-}lid^{ARID}$, and $UAS\text{-}lid^{C5HC2}$(from J. Secombe, Albert Einstein College of Medicine, Bronx, NY, USA) [refer to Materials and Methods in (Li et al., 2010) and

(Secombe, Li, 2007)], *Stat92E*^{06346} (from N. Perrimon, Harvard Medical School, Boston, MA, USA), *UAS-DE-Cad*^{DEFL} (from Y. Yamashita, University of Michigan, Ann Arbor, MI, USA), *UAS-Stat92E* (from E. Bach, New York University School of Medicine, New York, NY, USA), *UAS-Ptp61F shmiRNA* (Valium 20, TRiP.HMS00421 from Bloomington Stock Center, BL-32426), *Ptp61F*^{PBac} (Bloomington Stock Center, BL-17698), and *Act5C<stop<Gal4, UAS-GFP* (from J. Secombe, Albert Einstein College of Medicine, Bronx, NY, USA).

Clonal Induction

lid^{k06801} clones were generated using the FLP/FRT recombination system. The flies used had the following genotypes: *hs-FLP122; Ubi-GFP, Ubi-GFP, FRT40A/ lid*^{k06801} *FRT40A or hs-FLP122; Ubi-GFP, Ubi-GFP, FRT40A/ FRT40A.* Overexpression clones were generated using the FLP/FRT recombination system. The flies used had the following genotypes: *hs-FLP122; Act5C<stop<Gal4, UAS-GFP; UAS-lid or hs-FLP122; Act5C<stop<Gal4, UAS-GFP; UAS-lid*^{JmjC}. The clones were induced by heat shocking pupae on days 8 and 9 for 2 hrs at 37°C. After the second heat shock, flies were placed at 25°C and dissected and stained 3 days after clone induction.

Immunofluorescence Staining

Testes were dissected in 1XPBS and fixed in 4% formaldehyde for 30 minutes. For immunostaining, testes were incubated with primary antibodies overnight at 4 °C, followed by washes in 1XPBST and incubation with secondary antibodies for 2 hours at RT. The following primary antibodies were used: rabbit anti-Zfh1 (1:5000; from Ruth

Lehmann, Skirball Institute of Biomolecular Medicine, NY, USA); mouse anti-Armadillo [1:100; developed by Eric Wieschaus, Princeton University, Princeton, NJ, USA, and obtained from Developmental Studies Hybridoma Bank (DSHB)]; rat anti-Vasa (1:100; developed by Allan Spradling and Dianne Williams and obtained from DSHB); rabbit anti-Vasa (1:100; Santa Cruz, sc-30210); chicken anti-GFP (1:1000; Abcam, #13970); rabbit anti-Stat92E (1:800; from Denise Montell, Johns Hopkins School of Medicine, Baltimore, MD, USA); rabbit anti-phosphohistone H3 (Ser10) (1:2000; Millipore, #06-570); mouse anti-Bam (1:20; obtained from DSHB); mouse anti-α-spectrin (1:50; obtained from DSHB); mouse anti-FasIII (1:50; obtained from DSHB, 7G10); rabbit H3K4me3 (1:200; Cell Signaling, #9751S). Alexa 488, 568 and 633-conjugated Goat anti-mouse, anti-rabbit, and anti-rat secondary antibodies were used (1:200; Molecular Probes/Invitrogen).

Isolation of Total RNA and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT–PCR)

Total RNA was collected and isolated from wt and *lid* third instar larval testes using TRIzol reagent (Invitrogen, #15596-018) according to the manufacturer's instructions. The yield and quality of the RNA was determined using a NanoDrop spectrometer (NanoDrop Technology, San Diego, CA, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, #K1621). RNA Transcript levels were then measured using SYBR Green PCR Master Mix (Fermentas, #K0221) and normalized to *RpL32*. Sequences of primers used for qRT-PCR were as follows:

AAGGTGAGTGATTTGCTGTGCTGC (*Stat92E*-forward)

CAACAAGCGAGCATGAGAATGCCA (*Stat92E*-reverse)

CATGCTGCCCACCGGATTCAAGAAG (*RpL32*-forward)

CTCGTTCTCTTGAGAACGCAGGCGA (*RpL32*-reverse)

Western Blotting

Testes were dissected in 1XPBS. Samples were homogenized in 25 μl 1XPBS containing 4XDualColor Protein Loading Buffer (Fermentas, #R1011), boiled and loaded onto a 4-20% gradient SDS-PAGE gel (Novex, EC6065). Primary antibodies anti-H3K4me3 (1:1000; Cell Signaling, #9751S) and anti-H3 (1:5000; Abcam, ab1791) were used.

Fertility assay

Male and female virgin flies were collect at day 10 and single crosses were set up with either one male or female in each vial with two *yellow-white* (*y,w*) counterparts. The number of progeny produced in each vial was counted on day 10, 11, and 12. Controls were carried out using *y,w* male and female single crosses.

Statistical Analysis

Statistical significance was calculated using two-tailed Student's t-test or Fisher's test. P-values are indicated in figures or in figure legends. Error bars indicate standard deviation.

RESULTS

Lid demethylates H3K4me3 in the *Drosophila* testes

lid encodes a histone demethylase that has been shown to target H3K4me3 (Li et al., 2010). However, the role of Lid's demethylase function has not been tested specifically in *Drosophila* testes. To study the effect of *lid* loss on the level of H3K4me3 in testis, we used a strong loss-of-function allele of *lid* (lid^{10424}) (Gildea et al., 2000). We then generated lid^{10424}/Df hemizygous flies, referred to hereafter as *lid* flies. These *lid* flies are adult lethal, but a few escapers (2-3%) survive. These escapers are infertile and only survive for one or two days. Testes were dissected out of escapers and imaged using phase contrast microscopy. The testes were smaller in size compared to wt and the sperm was completely immotile. When looking at the third instar larval stage, *lid* mutant larvae are present at the expected Mendelian ratio when compared to their heterozygous sibling (data not shown). *lid* mutant pupae are also present, suggesting that these *lid* mutant flies failed to make the transition from pupae to adult flies. Because of the adult lethality, the histone demethylase activity of Lid in the fly testis was analyzed using third instar larvae. Western blotting experiments using H3K4me3 antibody showed significantly higher levels of H3K4me3 in *lid* testes compared to that of wt testes [relatively 4.03 times higher in *lid* testes compared to wt testes], consistent with Lid's H3K4me3-specific demethylase activity (Fig. 1A, 1B). We also used a heatshock activated FLP recombinase in combination with an *Act5C-Gal4* driver that is interrupted by a STOP cassette flanked by two FRT sites (Secombe et al., 2007), to drive transcription of a *UAS-lid* (Li et al., 2010) transgene to demonstrate that overexpression of *lid* in clones in the testis results in diminished levels of H3K4me3. Implementing a UAS/Gal4 binary system, we

heatshocked *UAS-lid* and *UAS-lid*^{JmjC} flies to generate GFP labeled clones that overexpressed *lid* and *lid*^{JmjC}, respectively. These germline clones showed decreased H3K4me3 signal compared to neighboring wt cells (Fig. 1C, C', C''). In addition to the *UAS-lid* transgene, a *UAS-lid* construct with a mutated JmjC domain, *UAS-lid*^{JmjC} (Li et al., 2010) was overexpressed. Clones overexpressing *UAS-lid*^{JmjC}, did not display the same decrease in levels of H3K4me3 seen in *UAS-lid* clones (Fig. 1D, D', D''). These data demonstrate that Lid acts as an H3K4me3 demethylase in the *Drosophila* testes. In addition, the JmjC domain is required for proper Lid demethylase function.

Loss of *lid* results in a decrease in GSC number

In order to determine the function(s) of *lid* in the male *Drosophila* GSC niche, we isolated testes from third instar larvae of *lid* mutant males and analyzed the niche architecture. We used antibodies against Armadillo (Arm) to label hub cells, zinc finger homeodomain 1 (Zfh1) to label cyst stem cells (CySCs) and early cyst cells, and Vasa to label germ cells (Leatherman and Dinardo, 2008). GSCs are identified as germ cells that are in direct contact with the hub (white arrows). Compared to wt testes, *lid* mutant testes have a significantly lower number of GSCs (Fig. 2B) [9.7±1.6 GSCs in wt testes (n=54) versus 6.0±1.0 GSCs in *lid* testes (n=81), $p<0.01$]. In addition, the GSCs in *lid* mutant testes appear to have a weaker association with the hub (Fig. 2C, 2D). This loss of GSCs could explain the infertility seen in the few adult escapers. These results suggest that Lid function is required in order to preserve GSCs around the hub.

Lid functions in germ cells to maintain GSC number in the niche

In order to determine in which cell type Lid is required to maintain GSC number, we performed a cell type-specific *lid* knockdown experiment. Different cell type-specific Gal4 drivers were used in combination with an *UAS-lid short hairpin microRNA* (*shmiRNA*) (Ni et al., 2011). *nanos (nos)-Gal4* (Van Doren et al., 1998) was used to knockdown of *lid* exclusively in germ cells, *unpaired (upd)-Gal4* (Boyle et al., 2007) to knockdown *lid* in hub cells, and *c587-Gal4* (Manseau et al., 1997) to knockdown *lid* in cyst cells. Only when we drove *UAS-lid shmiRNA* in germ cells did we see a significant decrease in GSC number comparable to the whole mutant (Figure 3A, 3B) [9.5±1.6 GSCs in *nos-Gal4* testes (n=27) versus 5.9±1.1 GSCs in *nos-Gal4>UAS-lid shmiRNA* testes (n=66), p<0.01]. Neither the hub cell driver *upd-Gal4* nor cyst cell driver *c587-Gal4* caused any significant change in GSC number (Fig. 3C) [9.1±1.6 GSCs in *upd-Gal4* testes (n=15) versus 9.2±1.3 GSCs in *upd-Gal4>UAS-lid shmiRNA* testes (n=31), p>0.05; 9.9±2.0 GSCs in *c587-Gal4* testes (n=16) versus 10.5±1.8 GSCs in *c587-Gal4>UAS-lid shmiRNA* testes (n=16), p>0.05]. These data demonstrate that Lid function is required in germ cells, but not in hub cells or cyst cells, to maintain GSC number.

The function of Lid in germ cells does not depend on its demethylase activity

As mentioned before, Lid shares domains with JARID1 family proteins, including the main catalytic JmjC domain. Recent studies have shown that this JmjC domain is required in order for Lid to properly function as a histone demethylase (Secombe et al., 2007). Our analysis using *UAS-lid* and *UAS-lid*^{JmjC} clones further confirms that the JmjC domain is essential for its H3K4me3-demethylase activity. In addition, Li et al. showed

that the JmjN domain is also necessary for Lid's H3K4me3 demethylase activity (Li et al., 2010). We chose to test three domains, JmjC, JmjN, and PHD3, for their ability to rescue the GSC loss phenotype in *lid* whole mutants. The PHD3 domain of Lid plays a role in binding to both H3K4me2 and H3K4me3 marks, with a consistent preference for the trimethylated mark (Li et al., 2010). Because we found Lid function to be required in germ cells to maintain GSCs around the hub, we used a *nos-Gal4* cell type-specific driver to express *UAS-lid, UAS-lid*^{JmjC}*, UAS-lid*^{JmjN}*,* and *UAS-lid*^{PHD3} in early germ cells. Quantifying the number of GSCs, we found that all four different transgenes were able to restore GSC number back to wt numbers (Fig. 3D) [10.9±2.0 GSCs in *nos-Gal4>UAS-lid lid* mutant testes (n=40); 11.1±1.8 GSCs in *nos-Gal4>UAS-lid*^{JmjC} *lid* mutant testes (n=38); 9.5±2.2 GSCs in *nos-Gal4>UAS-lid*^{JmjN} *lid* mutant testes (n=39); 8.9±2.9 GSCs in *nos-Gal4>UAS-lid*^{PHD3} *lid* mutant testes (n=38)]. Expressing *UAS-lid* in germ cells at *lid* mutant background served as a positive control as the full-length *lid* transgene should have the capacity to reverse the GSC loss phenotype. The *UAS-lid*^{PHD3} transgene should still be functional as a histone demethylase but can no longer bind to H3K4me2 or H3K4me3 marks (Li et al., 2010). Thus, we expected that expressing *UAS-lid*^{PHD3} in germ cells would also rescue the loss of GSCs. Most interesting, however, is that both the *UAS-lid*^{JmjC} and the *UAS-lid*^{JmjN} transgenes were able to restore GSC number in *lid* mutant testes. Both of these constructs have been shown to lack H3K4me3 demethylase activity (Li et al., 2010). These results suggest that Lid demethylase activity is not required in germ cells to maintain GSC number. Mutated JmjC, JmjN, and PHD3 domains did not have an impact on the ability of the *UAS-lid* transgene to rescue the GSC

loss. Thus, these three domains are dispensable for Lid's function in germ cells to maintain the GSC number in the niche.

Loss of *lid* results in premature differentiation of GSCs

In order to account for the loss of GSCs in *lid* mutant testes, we used antibodies against phosphorylated histone H3 (PH3), a marker for mitosis. Compared to wt, *lid* mutant GSCs were less mitotically active (Fig. 4A) [7.4% of GSCs (n=189) in wt testes were positive for PH3 versus 2.6% of *lid* GSCs (n=75), $p<0.01$]. This significant decrease in the mitotic index of GSCs in *lid* mutant testes could explain defects in *lid* males such as smaller testes and immotile sperm. However, less mitotic division is not a thorough explanation for the loss of GSCs.

In order to better understand the cause behind GSCs leaving the niche, we investigated the possibility of premature differentiation by first looking at spectrosome staining. Each GSC has a single spectrosome, which anchors the mitotic spindles during germ cell division (Fig. 4B). The GSCs divide asymmetrically to form a gonialblast, which then undergoes four rounds of mitotic division to form a sixteen-cell spermatogonial cyst. During this time, the spectrosome becomes a branched fusome, which connects all of the germ cells within a cyst to each other. When looking at *lid* testes, we found four-cell cysts that were directly associated with the hub (Fig. 4C). There are two possible explanations: the GSCs are prematurely differentiating and forming cyst next to the hub or spermatogonial cysts are homing back to the hub and dedifferentiating. In order to rule out dedifferentiation of spermatogonia back to GSCs, we did an assay for misoriented centrosomes. A *bona fide* GSC has two centrosomes, one

located towards the hub and the other directly away from the hub. In contrast, a dedifferentiated GSC has two centrosomes but both are oriented away from the hub, called misoriented centrosomes. However, when we compared the percentage of GSCs with misoriented centrosomes in wt testes versus *lid* mutant testes, there is not a significant difference (data not shown) [7.1% of GSCs in wt testes (n=104) had misoriented centrosomes versus 6.3% of GSCs in *lid* mutant testes (n=59), p>0.05]. This is consistent with the hypothesis that the niche is losing GSCs due to premature differentiation. We also performed immunostaining using antibodies against *Bam*, a differentiation marker that is expressed in four and eight-cell cysts but not earlier. In wt testes, germ cells staining positive for *Bam* are seen a distance away from the hub (Fig. 4D, D'). However, when we analyzed *lid* mutant testes, we saw *Bam*-expressing germ cells directly adjacent to the hub (Fig. 4E, E'). In summary, the presence of four-cell cysts and *Bam*-positive germ cells directly next to the hub suggests that the loss of GSCs is due to their premature differentiation.

Loss of *lid* causes a decrease in JAK-STAT signaling in the GSC niche

The JAK-STAT signaling pathway plays a prominent role in the proper maintenance of the GSC niche (reviewed by de Cuevas and Matunis, 2011). Specifically, Stat92E function in CySCs and early cyst cells is required for proper proliferation and maintenance of GSCs (Leatherman and DiNardo, 2010). Because of the influence of the JAK-STAT pathway on GSC maintenance, we investigated JAK-STAT signaling in *lid* mutant testes. In wt testes, we saw *Stat92E* all around the hub (Fig 5A). In the *lid* mutant testes, however, the *Stat92E* signal is absent, indicative of diminished JAK-STAT

signaling in *lid* testes (Fig. 5B). While immunostaining suggests that there is less JAK-STAT signaling, it does not specify the degree to which JAK-STAT signaling is reduced. In order to obtain a more quantitative measurement, we performed a quantitative reverse transcription polymerase chain reaction (qRT–PCR) using *Stat92E* primers (Fig. 5C). Results from the qRT–PCR also showed that the relative levels of *Stat92E* mRNA in *lid* mutant testes is less than half of the *Stat92E* level in wt testes [100±25 in wt testes relative to 49±43 in *lid* testes, $p<0.05$].

Because we also saw loss of GSCs when *lid* was knocked down only in germ cells, we hypothesized that JAK-STAT staining would also be decreased in germ cells in which *lid* was knocked down. When we knocked down *lid* in germ cells, immunostaining confirmed that levels of *Stat92E* were noticeably diminished. In the *nos-Gal4* control, *Stat92E* staining resembles the *Stat92E* staining pattern seen in wt testes (Fig. 5D), whereas in *nos-Gal4>UAS-lid shmiRNA* testes, the *Stat92E* signal was not present (Fig. 5E). These data show that loss of *lid* results in a loss of JAK-STAT signaling and, more specifically, Lid function is required in germ cells in order to preserve JAK-STAT signaling.

Loss of one copy of *Stat92E* in *lid* mutant testes results in a more severe phenotype

Because the loss of *lid* caused a loss of JAK-STAT signaling in the niche, we hypothesized that by removing one copy of *Stat92E* in the *lid* mutant background, we would be able to increase the severity of the phenotype. We used *Stat92E*^{06346}, a strong loss-of-function allele (Leatherman and DiNardo, 2008), in combination with our *lid* mutant flies in order to obtain the desired genotype. Immunostaining of testes showed

that all of the GSCs have disappeared and were replaced by spermatocytes, which were identified based on DAPI staining (data not shown). In a wt testis, the spermatocytes are on the end opposite to the niche. In our *lid* mutant with only one copy of *Stat92E*, these spermatocytes have aggregated around the hub and there are no GSCs present (Fig. 6A). The earliest stage in spermatogenesis seen in these testes is eight-cell spermatogonial cysts. These data further support that *lid* plays a role in preventing premature differentiation of GSCs via Stat92E because the additional loss of *Stat92E* dramatically increased the severity of the phenotype as all of the GSCs appear to have prematurely differentiated.

Expressing *Stat92E* in germ cells rescues GSC loss phenotype

We tested the ability of *UAS-Stat92E* (Bach et al., 2003) to rescue the GSC loss phenotype. Using the *nos-Gal4* driver, we expressed *UAS-lid shmiRNA* and *UAS-Stat92E* in germ cells and found that the GSC number was maintained (Fig. 6B, 6C) [10.0±1.1 GSCs in *nos-Gal4>UAS-Stat92E, UAS-lid shmiRNA* testes (n=44) versus 5.9±1.1 GSCs in *nos-Gal4>UAS-lid shmiRNA* testes (n=66), p<0.01]. We also expressed *UAS-Stat92E* in a wt background using *nos-Gal4* as a control to verify that the increased levels of Stat92E did not simply increase the number of GSCs, but rather Stat92E functioned to rescue the knockdown of *lid*. Interestingly, the number of GSCs in these testes was lower than our *nos-Gal4* control (Fig. 6C) [7.3±0.9 GSCs in *nos-Gal4>UAS-Stat92E* testes (n=16), p<0.01]. While we do not have an explanation for why overexpression of Stat92E in germ cells could result in loss of GSCs, this result does confirm that the ability of *Stat92E* to rescue GSC number is specific for testes in which *lid* has been knocked down

in germ cells. In summary, loss of one copy of *Stat92E* in the *lid* mutant background generates a more severe phenotype and restoring JAK-STAT signaling in flies that have *lid* knocked down in germ cells rescues the GSC loss phenotype.

Knockdown of *Ptp61F* prevents the decrease in GSC number

According to our model, loss of *lid* results in a decrease JAK-STAT signaling which in turn results in the loss of GSCs. One possible explanation for these outcomes is that Lid functions through an inhibitor of *Stat92E* by demethylating the H3K4me3 mark that regulates that gene. This led us to test the effects of knocking down the genes for the inhibitors of *Stat92E*. There are two known inhibitors of Stat92E in germ cells, *pias* and *Ptp61F* (Stine and Matunis, 2013). We chose to analyze the genetic interaction of *lid* with *Ptp61F*. First, using *Ptp61F*^{Pbac} (Thibault et al., 2004), a strong loss-of-function allele, we generated *lid* mutant flies that also had a defective copy of *Ptp61F*. When we immunostained the testes of these flies, we found that GSC number had been restored (Fig. 7A, 7B) [9.3±2.4 GSCs in *lid; Ptp61F*^{Pbac}/+ testes (n=10) versus 6.0±1.0 GSCs in *lid* testes (n=81), p<0.01]. In addition to *Ptp61F*^{Pbac} whole mutants, we also used the *nos-Gal4* driver to express *UAS-lid shmiRNA* and *UAS-Ptp61F shmiRNA* (Ni et al., 2011) in early germ cells. Knocking down *Ptp61F* only in germ cells also rescued the GSC loss phenotype (Fig. 7C, 7D) [9.7±1.6 GSCs in *nos-Gal4>UAS-lid shmiRNA, UAS-Ptp61F shmiRNA* testes (n=28) versus 5.9±1.1 GSCs in *nos-Gal4>UAS-lid shmiRNA* testes (n=66), p<0.01]. A *nos-Gal4>UAS-Ptp61F shmiRNA* control was also done, with no significant change in GSC number [9.6±2.1 GSCs in *nos-Gal4>UAS-Ptp61F shmiRNA*

testes (n=29), p>0.05]. Therefore, loss of *Ptp61F* can suppress the loss of GSC phenotype seen in both *lid* whole mutants and in germ cell specific knockdown of *lid.*

Expressing wt DE-Cadherin in germ cells prevents GSC loss in *lid* mutant

In order to further confirm the effect of loss of *lid* on JAK-STAT signaling, we attempted to rescue the loss of GSC phenotype by overexpression of the wt DE-Cadherin (*UAS-DE-Cad^{DEFL}*) (Inaba et al., 2010). In wt testes, GSCs are attached to the hub via DE-Cadherin mediated adherens junctions (Jenkins et al., 2003; Yamashita et al., 2003). In addition, *DE-Cadherin* is a downstream target of *Stat92E*, so if expressing *UAS-DE-Cad^{DEFL}* in germ cells prevents the loss of GSC in a *lid* mutant background, it will provide additional evidence that Lid functions to regulate JAK-STAT signaling in the niche. Indeed, when we drove *UAS-DE-Cad^{DEFL}* in *lid* mutant flies using the *nos-Gal4* driver for germ cells, we saw a rescue of the GSC loss phenotype (Fig. 7E, 7F) [6.0±1.0 GSCs in *lid* testes (n=81) versus 11.1±1.4 GSCs in *lid;nos-Gal4>DE-Cad^{DEFL}* testes (n=28), p<0.01]. In addition, we tested *nos-Gal4>DE-Cad* as a control in order to ensure that the overexpression of wild-type DE-Cadherin did not have a phenotype of its own. Indeed, there was no significant change in the number of GSC compared to wt [10.9±1.4 GSCs in *nos-Gal4>DE-Cad* testes (n=22) versus 9.7±1.6 GSCs in wt testes (n=54), p>0.05]. These data further supports the hypothesis that Lid is maintaining GSC number by regulating the JAK-STAT signaling pathway.

Loss of *lid* results in an enlarged hub

From our analysis of hub architecture in the *lid* testis, we observed an increased hub size in *lid* testes compared to that of wt testes (Fig. 8A) [114±28 μm^2 in wt testes (n=66) versus 231±84 μm^2 in *lid* testes (n=97), $p<0.01$]. We performed RNAi knockdown of *lid* using the three cell type-specific drivers, *upd-Gal4*, *c587-Gal4*, and *nos-Gal4*, in order to determine the cell autonomy of this phenotype. Much like the loss of GSC phenotype, only testes where *lid* is knocked down in germ cells displayed an enlarged hub (Fig. 8B) [129±35 μm^2 in *nos-Gal4* testes (n=27) versus 183±46 μm^2 in *nos-Gal4>UAS-lid shmiRNA* testes (n=66), $p<0.01$]. Neither knockdown in hub cells nor in cyst cells replicated this phenotype [134±14 μm^2 in *upd-Gal4* testes (n=15) versus 123±17 μm^2 in *upd-Gal4>UAS-lid shmiRNA* testes (n=31), $p>0.05$; 125±34 μm^2 in *c587-Gal4* testes (n=16) versus 137±24 μm^2 in *c587-Gal4>UAS-lid shmiRNA* testes (n=16), $p>0.05$]. Because the increase in hub size is only seen accompanying a loss of GSCs, it is possible that the enlarged hub is simply a secondary effect. It has been shown that loss of GSCs is often mirrored by an increase in hub size (Marthiens et al., 2010). As GSCs are lost, the healthy rosette around the hub deteriorates, allowing the hub to expand. In summary, loss of Lid function in germ cells results in an enlarged hub, although this could be a secondary effect to the loss of GSCs.

Lid functions in CySCs and early cyst cells to prevent overpopulation of Zfh1-positive cells around the hub and ectopic expression of Zfh1 in hub cells

Another phenotype that was seen in the *lid* whole mutant was the ectopic expression of Zfh1 in hub cells. In wt testes, Zfh1-postive CySCs envelop GSCs as they

form a ring one cell diameter away from the hub. Their nuclei are located away from the hub while thin protrusions extend toward the hub (Fig. 9A, A'). In *lid* mutant testes, however, Zfh1-expressing cells have their nuclei directly adjacent to the hub (Fig. 9B, white arrows). In addition, 61% of *lid* testes have hub cells that ectopically express Zfh1 (Fig. 9B', yellow arrows), whereas only 14% of wt testes exhibit this phenotype. After using the three different cell type-specific drivers, *upd-Gal4*, *nos-Gal4*, and *c587-Gal4*, to determine cell autonomy, we found that only when *lid* is knocked down in CySCs and early cyst cells is there overpopulation of Zfh1-postive cells around the hub and Zfh1 ectopically expressed in the hub (Fig. 9C, 9D, 9E) [13% of *c587-Gal4* testes (n=16) had Zfh1 signal inside the hub versus 69% of *c587-Gal4>UAS-lid shmiRNA* testes (n=16), p<0.01; in contrast to 20% of *upd-Gal4* testes (n=15) versus 17% of *upd-Gal4>UAS-lid shmiRNA* testes (n=31), p>0.05 and 17% of *nos-Gal4* testes (n=27) versus 14% of *nos-Gal4>UAS-lid shmiRNA* testes (n=66), p>0.05]. These data demonstrate that *lid* is required in CySCs and early cyst cells to prevent the ectopic expression of Zfh1 in the hub and the clustering of Zfh1-expressing cells around the hub.

However, because there was not a significant change in GSC number when *lid* was knocked down in CySCs and early cyst cells and the flies appeared to be healthy and fertile, this phenotype appears to be separate from the GSC loss. In addition, when *UAS-Zfh1* was ectopically expressed in the hub using *upd-Gal4*, a distinctive phenotype was not observed (Leatherman and DiNardo, 2008). Thus, we did not follow up on analyzing Lid function in CySCs and early cyst cells.

DISCUSSION

Lid is an H3K4me3 demethylase that is expressed in the male GSC niche

In this study, we analyzed the function of Lid, a histone demethylase that targets H3K4me3. There is another known H3K4me3 histone demethylase in *Drosophila*, dKDM2. Lid and dKDM2 have been shown to act redundantly to regulate H3K4me3 (Li et al., 2010), but data from transcriptome analysis in the testes indicates that *lid* is more highly expressed in the niche and gonialblasts whereas *dKDM2* levels are higher in spermatocytes. In addition, *lid* mRNA has been shown to be upregulated at the tip of the testis through RNA *in situ* hybridization (Gan et al., 2010). Because we focused on analyzing the germline stem cell (GSC) niche, we did not test for any genetic interactions between *lid* and *dKDM2*. Using a loss-of-function allele, we found that *lid* mutant flies only survived up until pupae stage and that the few escapers were sick and infertile. A western blot done on *lid* mutant fly testes against H3K4me3 antibody confirmed that Lid was functioning as an H3K4me3 demethylase in the testis. We also generated *UAS-lid* and *UAS-lid*^{JmjC} germ cell clones using a heatshock FLP/FRT system. Overexpressing *UAS-lid* and *UAS-lid*^{JmjC} using an *Actin-Gal4* driver revealed not only demonstrated Lid's demethylase activity in germ cells but also the necessity of a function JmjC domain in order for demethylation to occur.

We found that the *lid* testes were much smaller in size compared to wt testes. In addition, none of the sperm in the *lid* testis were motile, thus explaining the infertility of the escapers. When we analyzed the tip of the *lid* testis, we found certain niche defects. In our *lid* mutant testes, the hub was enlarged and Zfh1 was also ectopically expressed in the hub. However, the main defect that we saw was a significant loss of GSCs. This decrease

in the number of GSCs could explain the defects seen in *lid* sperm and therefore we decided to focus on analyzing this phenotype.

Lid function, but not demethylase activity, is required in germ cells to maintain GSCs

When we looked at the cell autonomy of the GSC loss phenotype, we found that expressing *UAS-lid shmiRNA* in germ cells generated a decrease in GSC number. However, when we used *upd-Gal4* and *c587-Gal4* to drive *UAS-lid shmiRNA* in the hub cells and cyst cells, respectively, we did not observe any significant change in GSC number. Therefore, we concluded that Lid function was only required in germ cells in order to maintain GSCs around the hub. We also assessed cell autonomy for the increase in hub size as well as the ectopic expression of Zfh1 in the hub. For these phenotypes, we found that Lid function in germ cells is required for maintaining wt hub size and Lid function in cyst cells is required for preventing hub cells from expressing Zfh1.

Because we knew that *lid* was required in germ cells in order to maintain GSCs around the hub, we followed up by driving different *UAS-lid* transgenes in germ cells using *nos-Gal4* to test for their ability to restore GSC number. The four transgenes that we used were *UAS-lid, UAS-lid^{JmjC}, UAS-lid^{JmjN},* and *UAS-lid^{PHD3}*. Studies have shown that the JmjC and JmjN domains are required for proper demethylase activity whereas the PHD3 domain functions to facilitate Lid binding to H3K4me2 and H3K4me3 marks on the histone tail. These four transgenes were expressed in early germ cells of *lid* mutant testes and all four were capable of rescuing the GSC loss phenotype. These data demonstrates that Lid's demethylase activity as well as its ability to bind onto the histone tail are not essential for its function of maintain GSCs around the hub. It is worth

mentioning that there are a total of seven different domains in the Lid protein, of which we only tested three. In addition, these domains may have functional redundancy, in which mutating single domains will not generate a noticeable defect. For future directions, investigating the four other domains as well as looking for genetic interactions between different combinations of domains would give more insight on Lid's functional structure.

GSCs are lost due to premature differentiation via the JAK-STAT pathway

After noticing that the *lid* mutant GSCs had a lower mitotic index compared to wt, we then stained for α-spectrin. Spectrosome staining revealed four-cell cysts that were in direct contact with the hub, which suggested premature differentiation. We also observed *Bam*-positive cells adjacent to the hub, further confirming our model. From past studies, the JAK-STAT pathway is known to play a major role in the proliferation and maintenance of GSCs. Therefore, we stained for *Stat92E* in *lid* mutant testes. Predictably, the JAK-STAT signaling was greatly diminished in the *lid* testes compared to wt testes. A recent study concluded that *Stat92E* function in CySCs is required for proper GSC maintenance but Stat92E in GSC does not promote self-proliferation (Leatherman and DiNardo, 2010). Our data contradicts their conclusion as we show that *lid* functions in GSCs to regulate JAK-STAT signaling, and loss of *lid* results in loss of GSCs. In addition to loss-of-function experiments, we also drove *UAS-Stat92E* using the *nos-Gal4* driver, which we found to restore the GSC number.

Lid represses *Ptp61F*, an inhibitor of Stat92E, to prevent premature differentiation

Lastly, we looked at upstream inhibitors of Stat92E in germ cells. We used *UAS-Ptp61F shmiRNA* as well as *Ptp61F*^{Pbac} to determine the effect of removing *Ptp61F* in *lid*

mutant testes. The GSC loss phenotype was rescued which leads us to believe that *lid* is regulating *Stat92E* through *Ptp61F*. While these data suggests that Lid could be functioning as an upstream regulator of *Ptp61F*, the mechanism of regulation is still unclear. After learning that the *UAS-lid, UAS-lid*^{JmjC}*, UAS-lid*^{JmjN}*,* and *UAS-lid*^{PHD3} transgenes all were able to rescue the loss of GSC phenotype; we concluded that Lid's H3K4me3 demethylase activity is not required for maintenance of GSCs in the niche. Thus, Lid must be regulating *Ptp61F* through a different means. Whether Lid directly interacts with *Ptp61F* cannot be concluded, but these data do suggest that *lid* is working upstream of *Ptp61F*. In addition, our data supports a derepression model where *lid* represses *Ptp61F* which in turn is an inhibitor of Stat92E. Therefore, loss of *lid* indirectly results in a loss of JAK-STAT signaling. However, the mechanism by which *lid* inhibits *Ptp61F* is still unknown. Another inhibitor of *Stat92E*, *pias*, has been identified in germ cells but we have not tested its genetic interaction with *lid*. For example, knockdown of *pias* in *lid* mutant flies could also restore the GSC number, in which case we would have to use different methods to determine if Lid was regulating *Ptp61F* or *pias*, or both.

In order to further support our model, we also looked at downstream targets of *Stat92E*, namely DE-Cadherin. We overexpressed *UAS-DE-Cad*^{DEFL} in germ cells of *lid* mutant testes and found that the number of GSCs had returned to wt numbers. Our study suggests that Stat92E in germ cells could be promoting GSC maintenance and proliferation. In addition, *lid* is a regulator this JAK-STAT signaling, possibly through the *Stat92E* inhibitor *Ptp61F* (Fig. 10). The JAK-STAT pathway plays a role in many different developmental processes and is particularly important for the development of the male GSC niche. By understanding the components of the signaling pathway, both

upstream and downstream, we could broaden our knowledge of how stem cells are regulated during development.

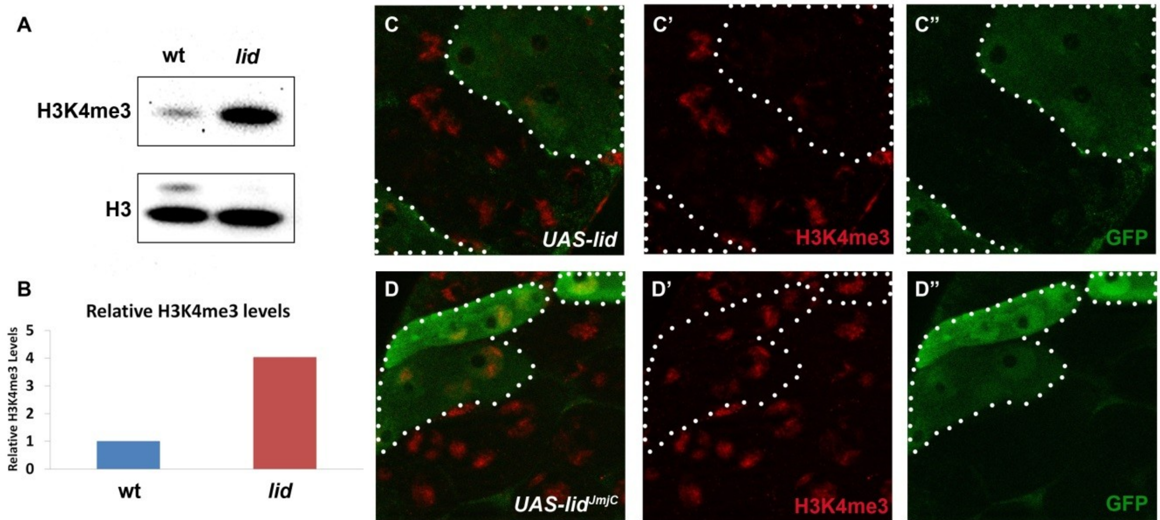

Figure 1. Lid functions as an H3K4me3 demethylase in the *Drosophila* testis. (A) Western blot using primary antibodies against H3K4me3 and H3 for wt and *lid* testes. **(B)** Quantification of western blot showing relative levels of H3K4me3 after normalizing with H3 control in *lid* testes versus wt testes. **(C-D'')** Immunostaining using antibodies against H3K4me3 (red) and GFP (green) of (C, C', C'') *UAS-lid* clones and (D, D', D'') *UAS-lid^{JmjC}* clones. GFP positive clones are outlined (white dotted line).

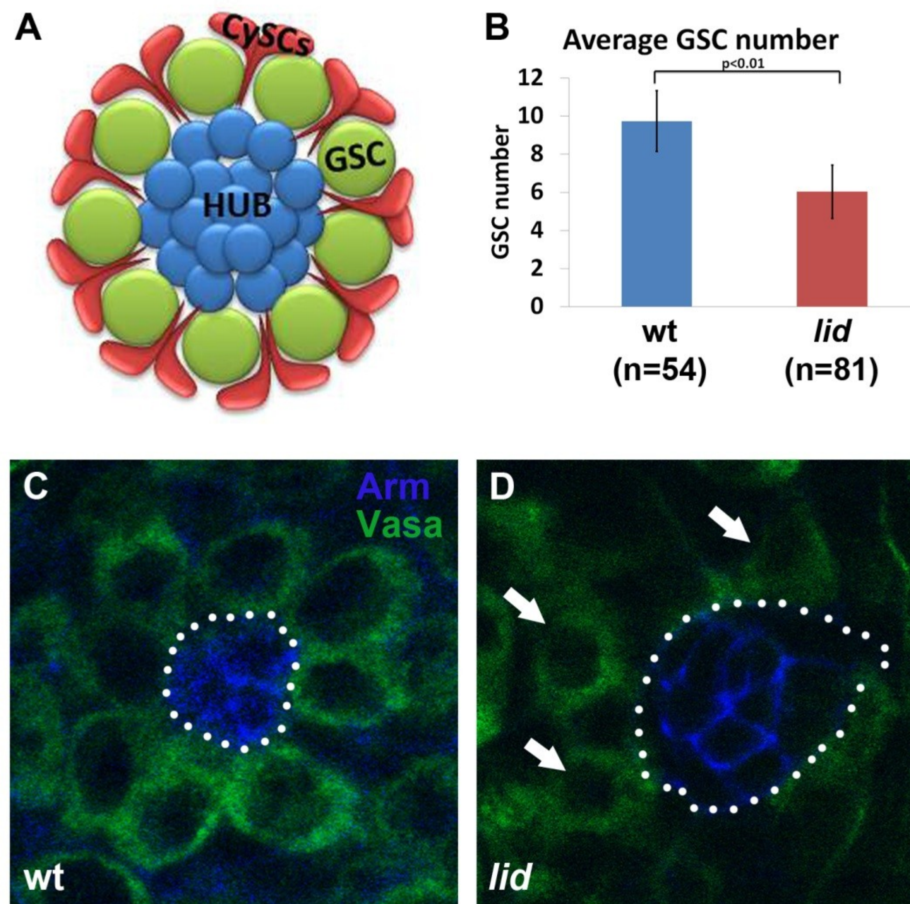

Figure 2. Loss of *lid* decreases GSC number. (A) Schematic of the *Drosophila* testis niche. CySCs, cyst stem cells; GSC, germline stem cell. **(B)** Quantification of average number of GSCs in wt and *lid* testes. P-value calculated using Student's t-test. Error bars represent s.d. **(C, D)** Immunostaining using antibodies against Arm (blue) and Vasa (green) in (C) wt and (D) *lid* testes. Arrows point to GSCs. Hub area is outlined (white dotted line).

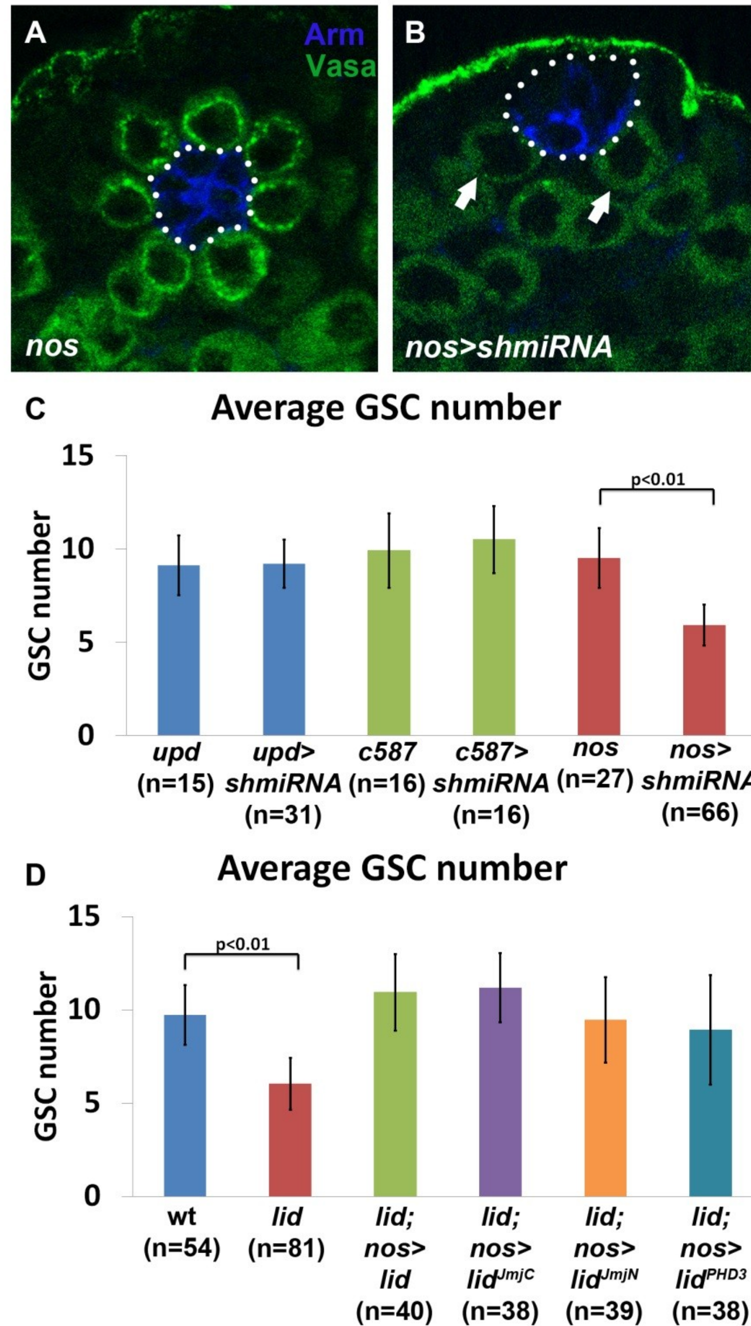

Figure 3. Lid function, but not demethylase activity, is required in GSCs to maintain GSC number. (A, B) Immunostaining using antibodies against Arm (blue) and Vasa (green) in (A) *nos-Gal4* and (B) *nos-Gal4>UAS-lid shmiRNA* testes. Arrows point to GSCs. Hub area is outlined (white dotted line). **(C)** Quantification of average number of GSCs for *UAS-lid shmiRNA* knockdown. **(D)** Quantification of average number of GSCs for *UAS-lid*, *UAS-lid*^{JmjC}, *UAS-lid*^{JmjN}, and *UAS-lid*^{PHD3} rescue. P-value calculated using Student's t-test. Error bars represent s.d.

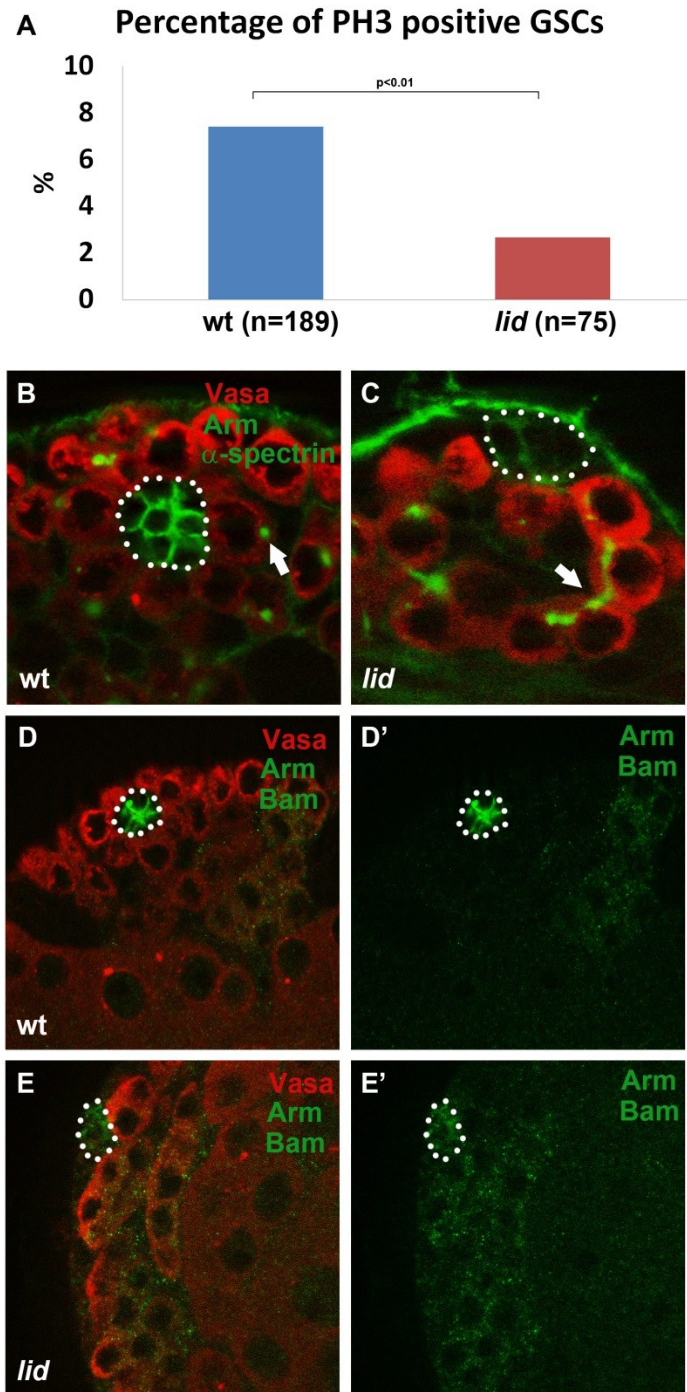

Figure 4. Loss of *lid* leads to premature differentiation of GSCs. (A) Percentage of PH3 postive GSCs. P-value calculated using Fisher's test. **(B, C)** Immunostaining using antibodies against Arm (green), α-spectrin (green), and Vasa (red) in (B) wt and (C) *lid* testes. Arrows point to spectrosome (B) and branched fusome (C). **(D-E')** Immunostaining using antibodies against Arm (green), Bam (green), and Vasa (red) in (D, D') wt and (E, E') *lid* testes. Hub area is outlined (white dotted line).

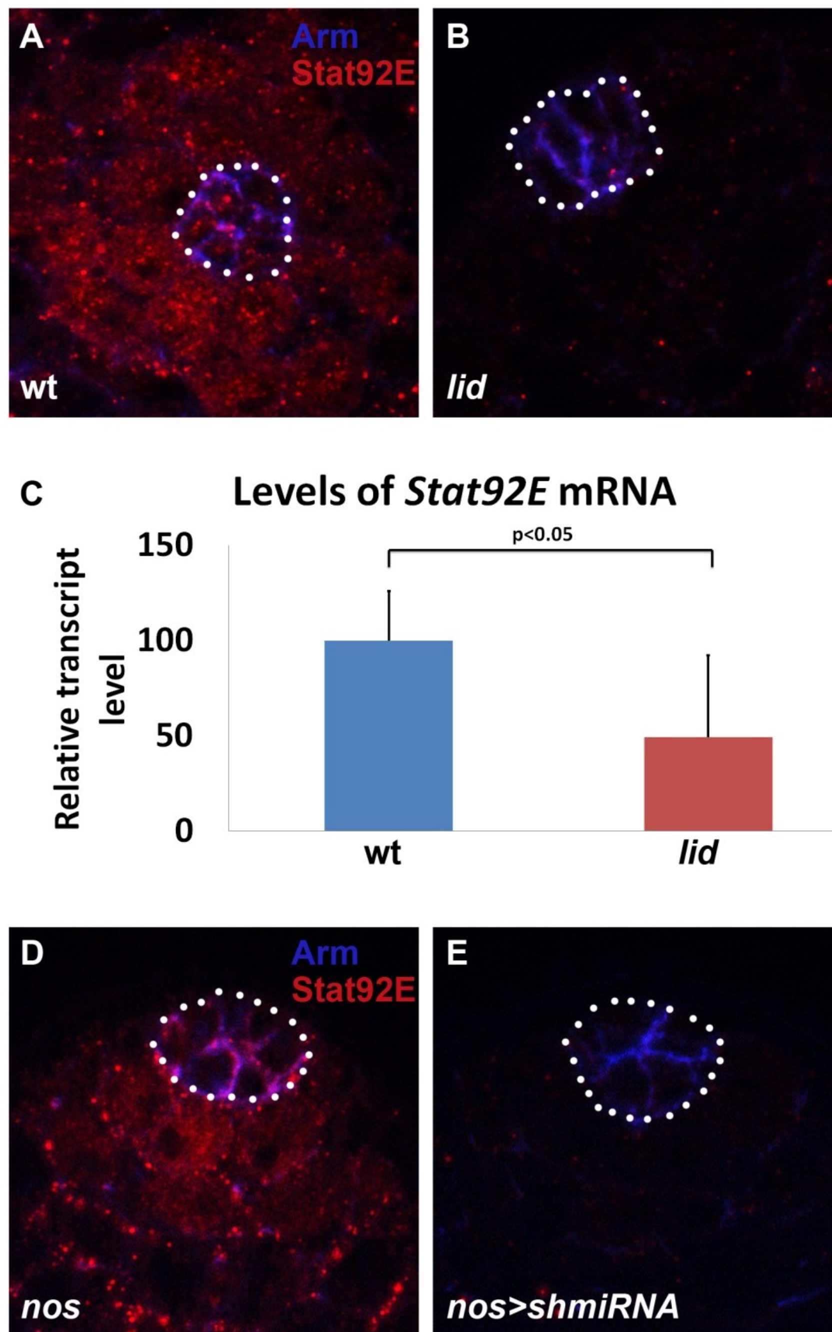

Figure 5. Loss of *lid* decreases JAK-STAT signaling. (A, B) Immunostaining using antibodies against Arm (blue) and Stat92E (red) in (A) wt and (B) *lid* testes. **(C)** *Stat92E* mRNA measured by qRT-PCR in three independent biological replicates, normalized by *RpL32*. P-value calculated using Student's t-test. Error bars represent s.d. **(D, E)** Immunostaining using antibodies against Arm (blue) and Stat92E (red) in (D) *nos-Gal4* and (E) *nos-Gal4>UAS-lid shmiRNA* testes. Hub area is outlined (white dotted line).

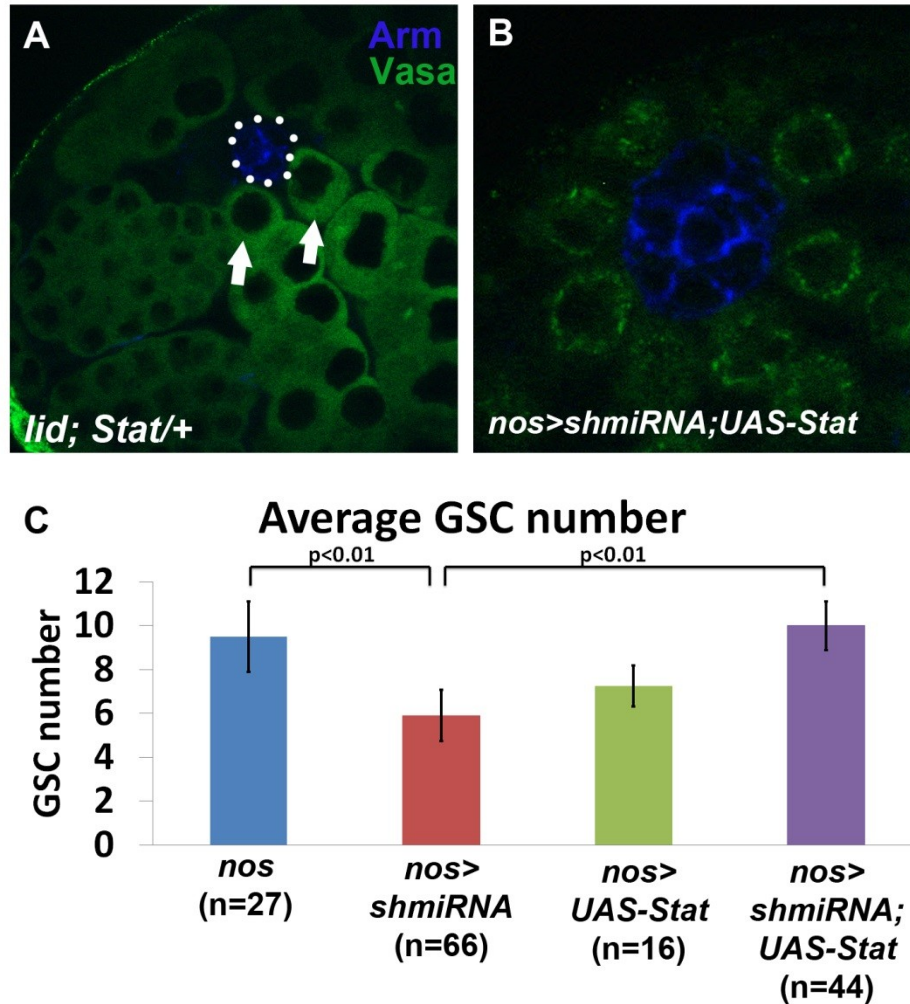

Figure 6. Loss of *Stat92E* enhances phenotype whereas overexpression of *Stat92E* restores GSC number. (A, B) Immunostaining using antibodies against Arm (blue) and Vasa (green) in (A) *lid;Stat92E*^{06346}/+ and (B) *nos-Gal4>UAS-lid shmiRNA;UAS-Stat92E* testes. Arrows point to spermatocytes next to the hub. Hub area is outlined (white dotted line). **(C)** Quantification of average number of GSCs for *nos-Gal4>UAS-lid shmiRNA;UAS-Stat92E* rescue. P-value calculated using Student's t-test. Error bars represent s.d.

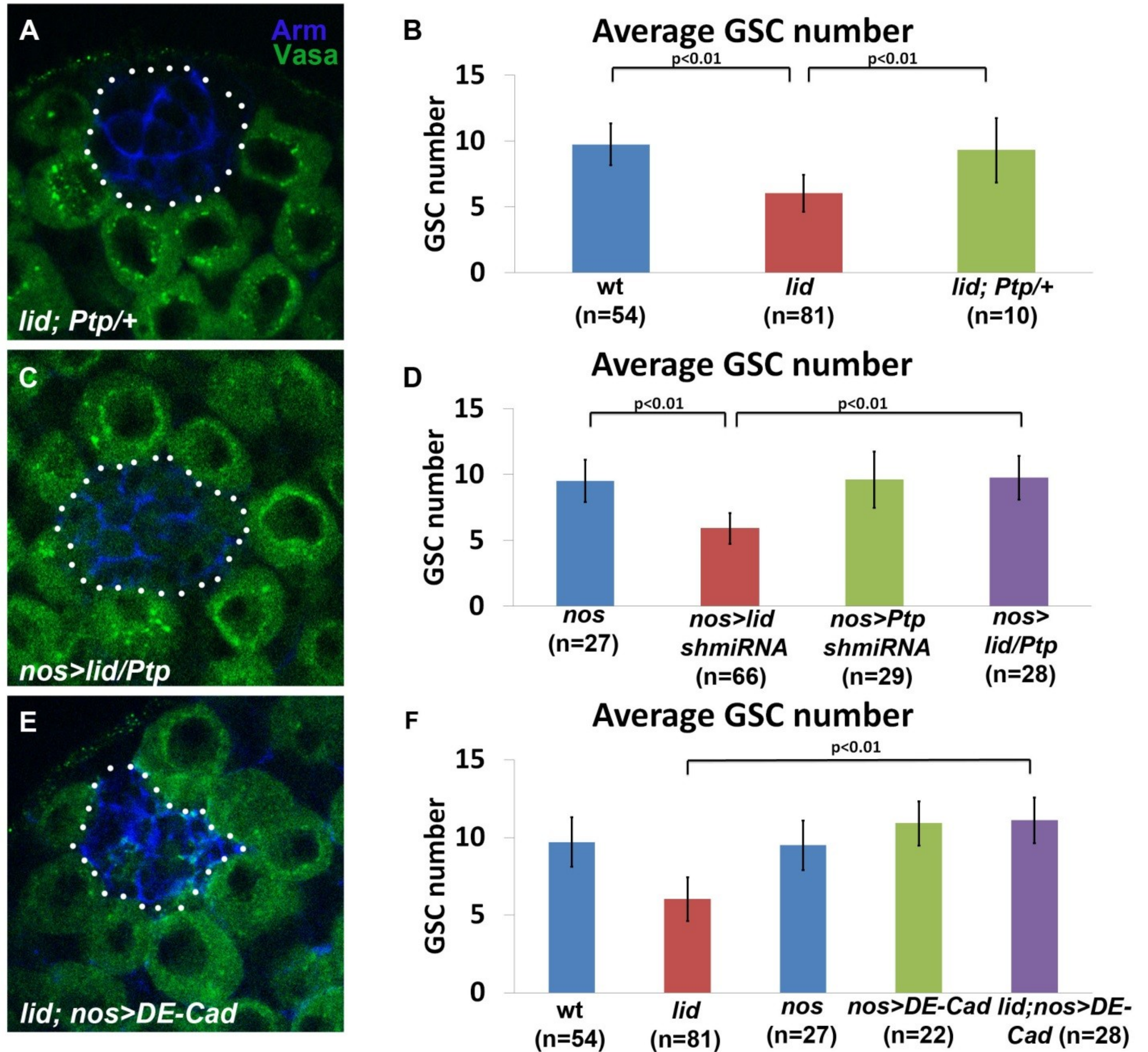

Figure 7. Loss of *Ptp61F* rescues GSC loss in *lid* mutants. (A) Immunostaining using antibodies against Arm (blue) and Vasa (green) in *lid;Ptp61F^{Pbac}/+* testes. **(B)** Quantification of average number of GSCs for *lid;Ptp61F^{Pbac}/+* rescue. **(C)** Immunostaining using antibodies against Arm (blue) and Vasa (green) in *nos-Gal4>UAS-lid shmiRNA;UAS-Ptp61F shmiRNA* testes. Hub area is outlined (white dotted line). **(D)** Quantification of average number of GSCs for *nos-Gal4>UAS-lid shmiRNA;UAS-Ptp61F shmiRNA* rescue. **(E)** Immunostaining using antibodies against Arm (blue) and Vasa (green) in *lid;nos-Gal4>DE-Cad* testes. **(F)** Quantification of average number of GSCs for *lid;nos-Gal4>DE-Cad* rescue. P-value calculated using Student's t-test. Error bars represent s.d.

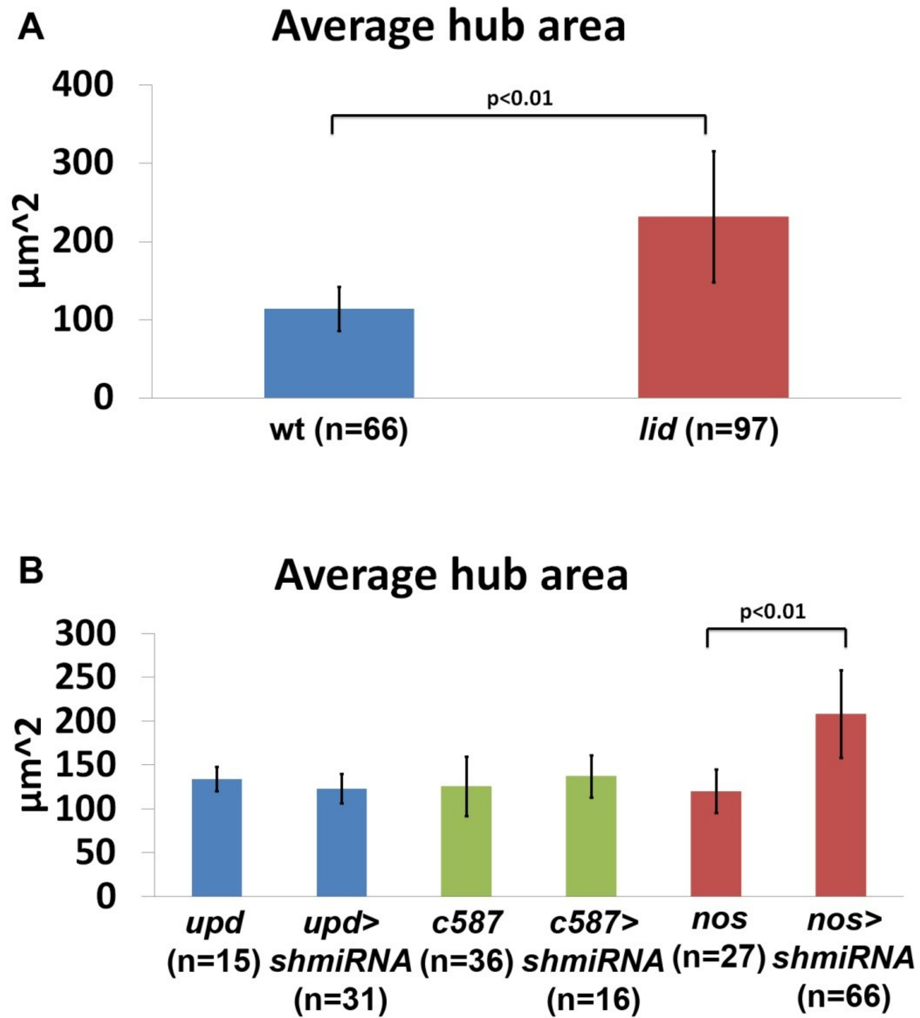

Figure 8. Lid functions in germ cells to prevent expansion of the hub. (A) Quantification of average hub area for wt and *lid* testes. **(B)** Quantification of average hub area for *UAS-lid shmiRNA* knockdown. P-value calculated using Student's t-test. Error bars represent s.d.

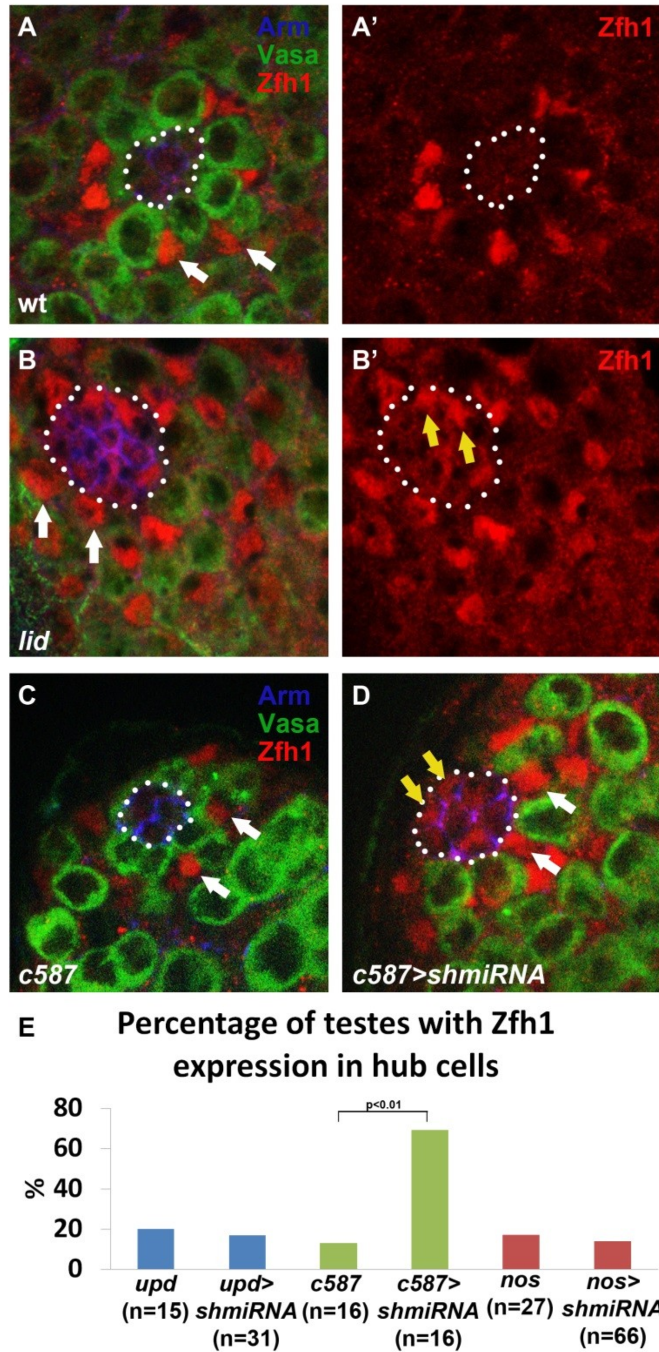

Figure 9. Lid functions in CySCs and/or early cyst cells to repress overpopulation of Zfh1-expressing cells around the hub and ectopic Zfh1 expression in hub cells. (A-D) Immunostaining using antibodies against Arm (blue), Vasa (green), and Zfh1 (red) in (A, A') wt, (B, B') *lid*, (C) *c587-Gal4*, and (D) *c587-Gal4>UAS-lid shmiRNA* testes. White arrows point to Zfh1-expressing cells with nuclei that directly contact the hub. Yellow arrows point to hub cells ectopically expressing Zfh1. Hub area is outlined (white dotted line). **(E)** Quantification of percentage of testes with ectopic Zfh1 expression in hub cells for *UAS-lid shmiRNA* knockdown. P-value calculated using Student's t-test. Error bars represent s.d.

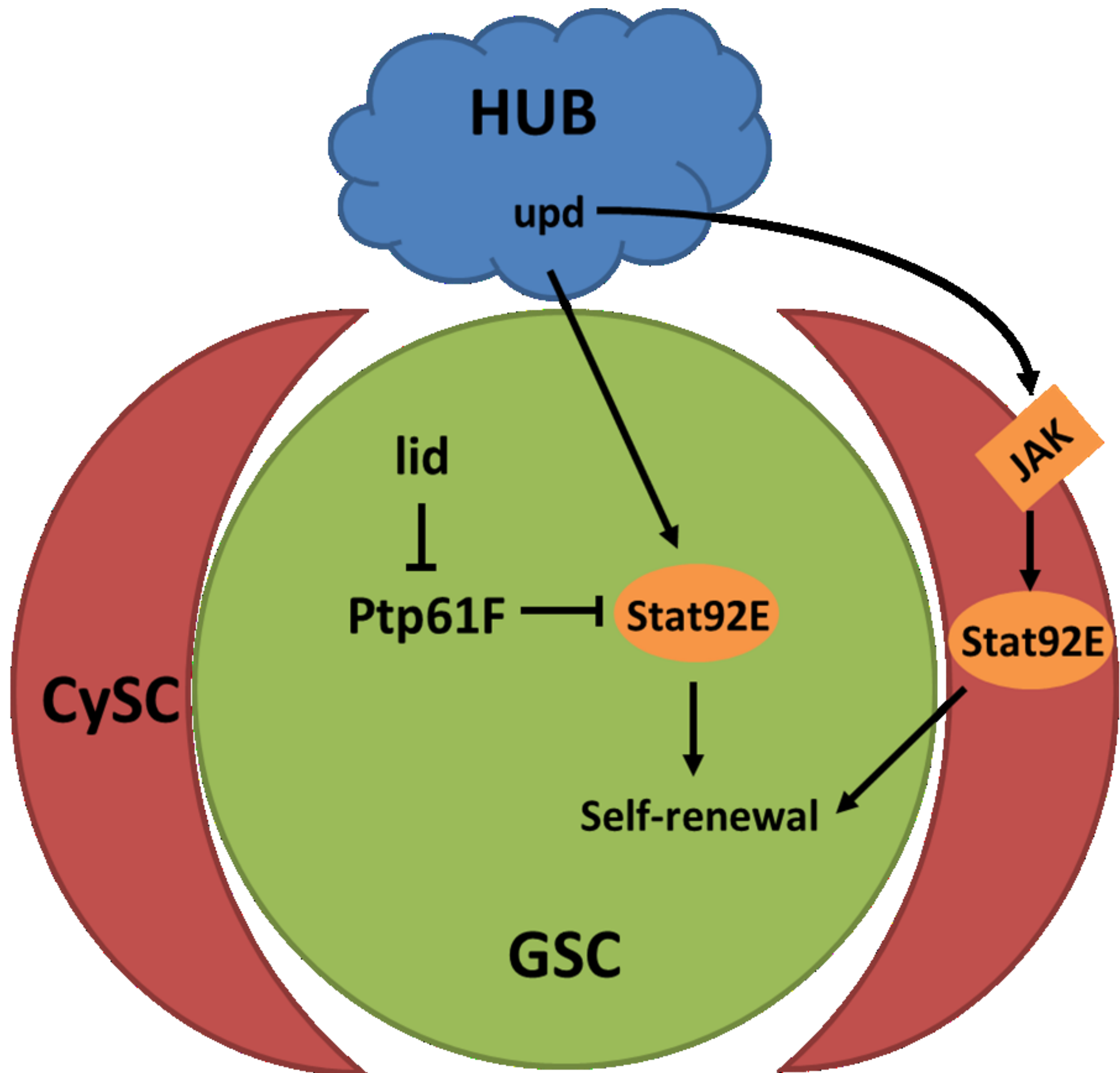

Figure 10. Model of Lid function in the *Drosophila* testis niche. Lid positively regulates the JAK-STAT signaling pathway in GSCs through a derepression mechanism. Lid represses *Ptp61F*, a Stat92E inhibitor in GSCs, which in effect upregulates *Stat92E* expression (see Discussion for details). GSC, germline stem cell; CySC, cyst stem cell.

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YUPING DEREK LI

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EDUCATION

Johns Hopkins University Graduate School Baltimore, MD
Masters of Science: Molecular and Cellular Biology Graduation Date: May 2014
Cumulative GPA: 4.00

Alumnus, Johns Hopkins University Baltimore, MD
Zanvyl Krieger School of Arts and Sciences Graduation Date: May 2013
Majors: Honors Molecular and Cellular Biology, Neuroscience
Minor: Entrepreneurship and Management
Cumulative GPA: 3.94

AWARDS/ACHIEVEMENTS

Dean's List, Johns Hopkins University 2010 – 2013

Danny Lee Award for Outstanding Undergraduate Research May 2013

EXPERIENCE

Dr. Xin Chen's Lab at Johns Hopkins University
Principal Investigator: Dr. Xin Chen
Research Assistant Aug 2010 – May 2013

- Worked on characterizing and analyzing the function of a histone demethylase, lid, in *Drosophila*. Presented findings at lab meetings every two months.
- Presented a poster at Germ Cells meeting at Cold Spring Harbor, New York (Oct 2012).
- Gained skills in common lab techniques (PCR, Genetic crossing, Gel electrophoresis, etc.).

Mouse Tri-Lab at Johns Hopkins University
Principal Investigator: Dr. Haiqing Zhao
Research Assistant Jan 2013 – June 2013

- Worked on uncovering the function of a novel protein using GST-pull down assay.

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Health Leads at Harriet Lane Clinic
Supervisor: Efua Opoku
Advocate, Data committee, Resource team May 2012 – Present

- Connected clinic patients and their families to community resources (i.e. food stamps, career centers, etc.).
- Tracked overall data for Health Leads at Harriet Lane Clinic and presented a poster at Undergraduate Conference of Public Health at Johns Hopkins University.

Johns Hopkins University
Instructors: Dr. Stewart Hendry and Dr. Haiqing Zhao
Undergraduate Teaching Assistant Aug 2012 – May 2013

- Lead review sessions, tutored students, wrote exam questions, and graded exams.

Instructor: Dr. Rebecca Pearlman
Graduate Teaching Assistant Aug 2013 – Present

- Set up and instructed weekly labs for General Biology course.

Global Brigades – Water Brigades
Volunteer June 2011

- Set up water distribution system for a village in Honduras.
- Worked with local residents to create a presentation for the children to teach them about the importance of clean water.

AFFILIATIONS/MEMBERSHIPS

Phi Beta Kappa May 2013 – Present

Nu Rho Psi Honors Society Aug 2012 – May 2013

Beta Beta Beta Honors Society May 2012 – May 2013