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A Regulatory Network of *Drosophila* Germline Stem Cell Self-Renewal

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SUMMARY

Stem cells possess the capacity to generate two cells of distinct fate upon division: one cell retaining stem cell identity and the other cell destined to differentiate. These cell fates are established by cell-type-specific genetic networks. To comprehensively identify components of these networks, we performed a large-scale RNAi screen in *Drosophila* female germline stem cells (GSCs) covering ~25% of the genome. The screen identified 366 genes that affect GSC maintenance, differentiation, or other processes involved in oogenesis. Comparison of GSC regulators with neural stem cell self-renewal factors identifies common and cell-type-specific self-renewal genes. Importantly, we identify the histone methyltransferase Set1 as a GSC-specific self-renewal factor. Loss of *Set1* in neural stem cells does not affect cell fate decisions, suggesting a differential requirement of H3K4me3 in different stem cell lineages. Altogether, our study provides a resource that will help to further dissect the networks underlying stem cell self-renewal.

INTRODUCTION

Stem cells play essential roles during animal development and homeostasis. Embryonic stem cells develop into all types of tissues and organs, whereas adult stem cells continuously replace dying and damaged cells. One of the key questions in stem cell biology is to understand the molecular basis of how stem cell self-renewal is controlled. Although mammalian cell culture approaches have provided insight in this process (Ding et al., 2009; Hu et al., 2009), it is desirable to study stem cells in their native environment.

Drosophila germline stem cells (GSCs) are a model of choice to identify genes involved in stem cell self-renewal (Spradling et al., 2011; Xie et al., 2008). In the *Drosophila* ovary, two or three GSCs are located in the most anterior part of the germarium, where they interact with the stem cell niche. A GSC divides asymmetrically to produce another self-renewing GSC and a cystoblast (CB) committed to differentiate. The CB divides four times synchronously to form a 16-cell cyst. Of these, one cell will differentiate into an oocyte, whereas the remaining cells will adopt a nurse cell fate. The activity of GSCs is controlled both by extrinsic and intrinsic factors. Decapentaplegic (Dpp) and Glass bottom boat (Gbb) produced from niche activate bone morphogenetic protein (BMP) signaling in the GSC to repress the transcription of a key differentiation gene, *bag of marbles* (*bam*), thereby maintaining GSC identity (Chen and McKearin, 2003; McKearin and Spradling, 1990; Song et al., 2004; Xie and Spradling, 1998). Besides cell-to-cell signaling, stem cell intrinsic programs are important for binary fate decisions. Nanos and Pumilio, components of a translational repression complex, are important for GSC maintenance (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Wang and Lin, 2004). Similarly, components of the microRNA machinery are required for GSC maintenance (Förstemann et al., 2005; Jin and Xie, 2007; Park et al., 2007; Yang et al., 2007), suggesting that translational control is essential to maintain stem cell identity.

GSC self-renewal and differentiation are further controlled at the level of chromatin structure, transcription, and splicing. The chromatin-remodeling factor Iswi and the DNA-associated protein Stonewall are required for GSC maintenance through *bam*-dependent and -independent pathways (Maines et al., 2007; Xi and Xie, 2005). Similarly, Scrawny (Scny), a histone (H2B) deubiquitinase (Buszczak et al., 2009), and the histone H3K9 trimethylase Eggless (Egg) have been shown to be required for GSC maintenance (Wang et al., 2011). Conversely, the female-specific RNA-binding protein Sex-lethal (Sxl), as well as the U1 snRNP protein Sans-fille (Snf) that controls *sxl* alternative splicing, is essential for GSC differentiation (Chau et al., 2009; Schüpbach, 1985) in part through regulation of Nanos levels (Chau et al., 2012).

Historically, genes regulating GSCs have been identified via genetic screens for female sterility in homozygous mutant animals (Cooley et al., 1988; Perrimon et al., 1986; Schüpbach

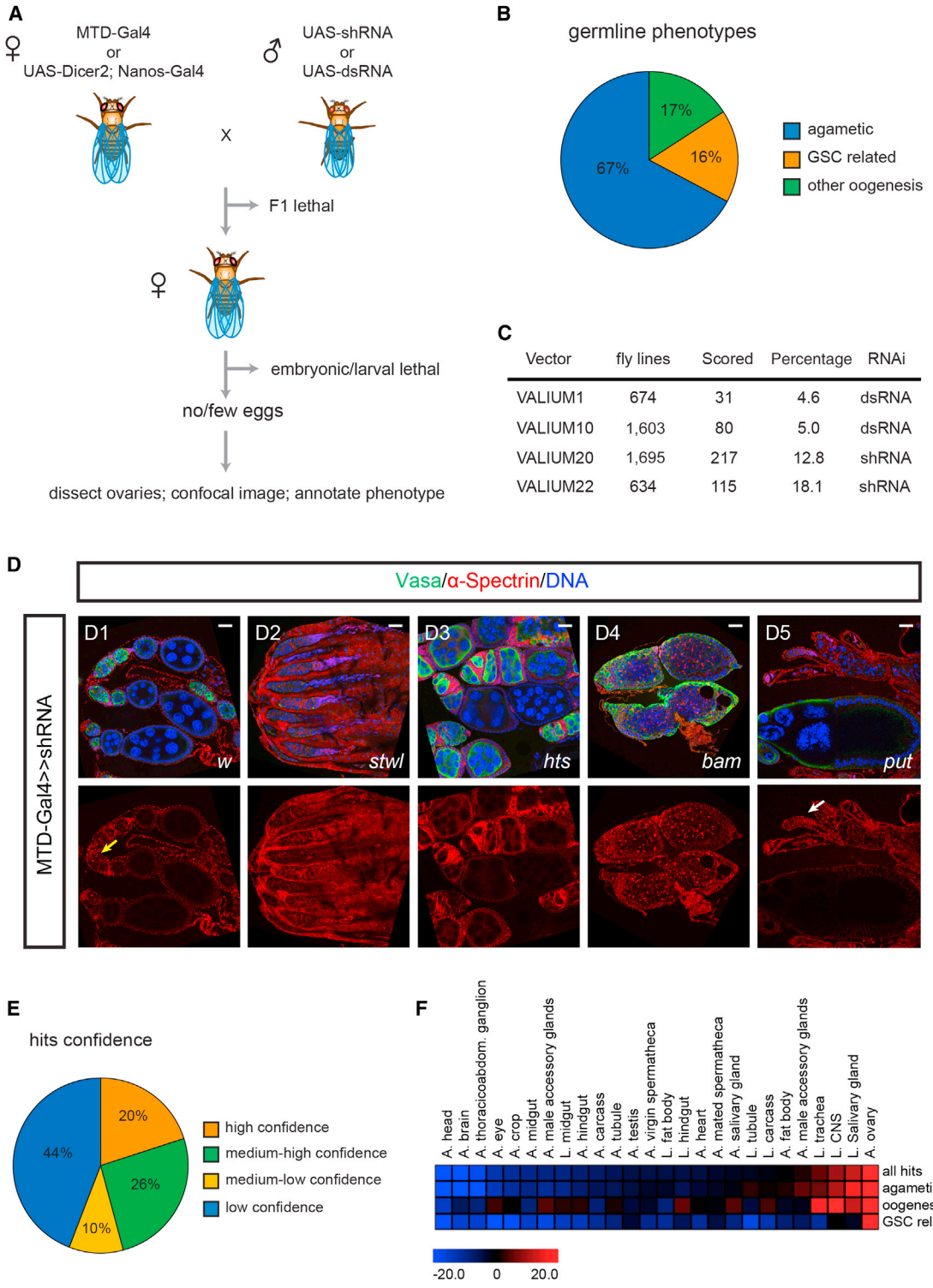


Figure 1. Transgenic RNAi Screen

(A) Workflow of the germline RNAi screen.

(B) F1 females with no eggs or few eggs were dissected, and ovaries were analyzed by confocal microscopy. The phenotypes were divided into three categories: agametic, GSC related, and other oogenesis.

(C) Summary of the screen results.

(D) Ovaries expressing shRNAs targeting *w* (D1), *stwl* (D2), *hts* (D3), *bam* (D4), or *put* (D5) by *MTD-Gal4* stained for α-Spectrin, Vasa, and DAPI. Yellow arrow indicates GSCs. White arrow points to empty germarium. Scale bars, 20 μm.

(legend continued on next page)

and Wieschaus, 1991). However, most genes relevant to oogenesis are also required during animal development, making it impossible to recover homozygous mutant animals. Although the phenotypes of these genes can be analyzed by clonal mosaic analysis approaches, as done for maternal effect phenotypes (Perrimon et al., 1989, 1996), systematic screens for GSC self-renewal and differentiation have not been done. Recently, transgenic RNAi in *Drosophila* has been widely used to study gene function in somatic tissues, including other stem cell systems such as neuroblasts (Nbs) (Dietzl et al., 2007; Neumüller et al., 2011). Here, we systematically analyzed GSC self-renewal using transgenic RNAi optimized for germline expression (Ni et al., 2009, 2011). We screened a collection of 3,491 germline-enriched genes and identified 366 that cause female fertility defects, allowing us to construct a network of the genes regulating GSC self-renewal. Cross-correlation with regulators of Nb self-renewal revealed GSC-specific as well as commonly required regulators of self-renewal. We demonstrate a specific role for the histone methyltransferase Set1 in GSCs and identify *scny* and *domino* (*dom*) as commonly required regulators in GSCs and Nbs. Our data thus constitute a useful resource for future studies of stem cell self-renewal.

RESULTS

GSC Self-Renewal Screen

To systematically analyze the function of individual genes in the female germline, we screened the existing TRiP (Transgenic RNAi Project) collection of long double-stranded RNA (dsRNA) (VALIUM 1 and 10 vectors) and short small hairpin RNA (shRNA) (VALIUM20 and 22 vectors) lines (Ni et al., 2009, 2011). To express shRNAs or dsRNAs, we used a maternal triple-driver *MTD-Gal4* or *UAS-dcr2; nanos-Gal4* to produce strong expression in the germarium and throughout oogenesis (Figures 1A and 2A) (Petrella et al., 2007). To identify potential stem cell phenotypes, ovaries of F1 females that laid no eggs were dissected and stained for three markers: the α-Spectrin antibody labels the spectrosome and the fusome, cytoplasmic organelles present in stem cells and cystocytes, respectively; the Vasa antibody labels all germ cells; and DAPI was used to label nuclei for monitoring oocyte and nurse cell formation (Figure 1D1). We took confocal images of the ovaries, annotated the phenotypes, and integrated all information into an online database (http://www.flyrnai.org/RSVP.html).

In total, we screened 4,608 transgenic lines, representing 3,491 germline-enriched genes or ~25% of the *Drosophila* genome (Table S1 available online). Among them, 444 lines, targeting 366 genes, showed oogenesis defects. The phenotypes were divided into three categories: agametic (67%), GSC related (16%), and other oogenesis (17%) (Figure 1B). In agametic ovaries, no or very few Vasa-positive germ cells are present, suggesting a defect in cell survival or GSC maintenance (Figure 1D2). GSC-related phenotypes include those that block stem cell differentiation and those that show bona fide stem cell maintenance phenotypes. For example, *bam shRNA* ovarioles were filled with extra stem cell-like cells (Figure 1D4) (McKearin and Spradling, 1990). In *punt* (encoding the type II transforming growth factor β [TGF-β] receptor) *shRNA* ovaries, GSCs were lost from the germarium, and differentiating egg chambers could be observed at later stages of oogenesis (Figure 1D5) (Xie and Spradling, 1998). Finally, many lines were associated with other oogenesis defects, such as fusome structure (Figure 1D3), oocyte fate specification (Figure S1), nurse cell number, oocyte nuclear localization, and egg polarity.

Quality Control

Four lines of evidence suggest that our screen has identified stem cell regulators with high confidence. First, we found many previously identified genes required for GSC differentiation (*bam*, *sxl*, *otu*, *Mei-P26*, *mael*, *twin*, and *aret*) and GSC maintenance (*punt*, *dcr-1*, *iswi*, *scny*, *stwl*, and *egg*) (Figure S1) (Buszczak et al., 2009; Findley et al., 2003; Jin and Xie, 2007; Maines et al., 2007; McKearin and Spradling, 1990; Morris et al., 2005; Page et al., 2000; Parisi et al., 2001; Pek et al., 2009; Schüpbach, 1985; Wang et al., 2011; Xi and Xie, 2005; Xie and Spradling, 1998). Second, many of the identified hits (96 genes) whose gene products are subunits of protein complexes show a high degree of phenotypic similarity (see below). Third, we were able to confirm the phenotype of 73 genes by two independent RNAi lines (Figure 1E; Table S1). Finally, we obtained evidence for efficient knockdown of a select set of genes by quantitative PCR (qPCR) analyses or antibody staining.

The set of genes associated with GSC phenotypes is significantly enriched for genes expressed in adult ovaries and in the larval CNS, whereas most other tissues are underrepresented (Figure 1F). Because both tissues contain stem cells and differentiated cell types, they might use similar molecular mechanisms to regulate self-renewal. Human orthologs were found for 96% of our identified gene set, suggesting that those genes might have conserved functions in mammalian stem cell systems (Table S1).

Among our identified RNAi lines, agametic phenotypes (67%) represent the largest category. Those genes could be required for general cell viability, or they might have specific roles in stem cell maintenance. To distinguish these possibilities, we screened 174 agametic lines using *maternal-tub-Gal4* (*MAT-Gal4*), which induces transgene expression outside the GSC compartment starting from stage 1 egg chambers in the posterior germarium (Figure 2A). Thus, this experiment can distinguish cell-essential genes from GSC regulators. For example, *Nxt1 shRNA* is agametic with *MTD-Gal4*, and is also defective in oogenesis with *MAT-Gal4*, suggesting a general requirement in cell survival (Figure 2B). However, *Top2 shRNA* is agametic with *MTD-Gal4* but produces normal eggs with *MAT-Gal4*,

(E) Confidence of identified 366 genes from the screen. High-confidence genes are identified by two or more independent RNAi lines. Medium-high confident genes are identified by one RNAi line, but they cocomplex with high-confidence hits. Medium-low confidence genes are identified by one RNAi line, but they cocomplex with other low-confidence hits. Low-confidence hits are identified by one RNAi only.
(F) Heatmap showing over- and underrepresentation of tissue-specific gene sets (as defined by their expression levels in the listed tissues) in three phenotypic categories found in the screen (B). Blue, underrepresented; red, overrepresented. A, adult; L, larvae.
See also Tables S1 and S2 and Figure S1.

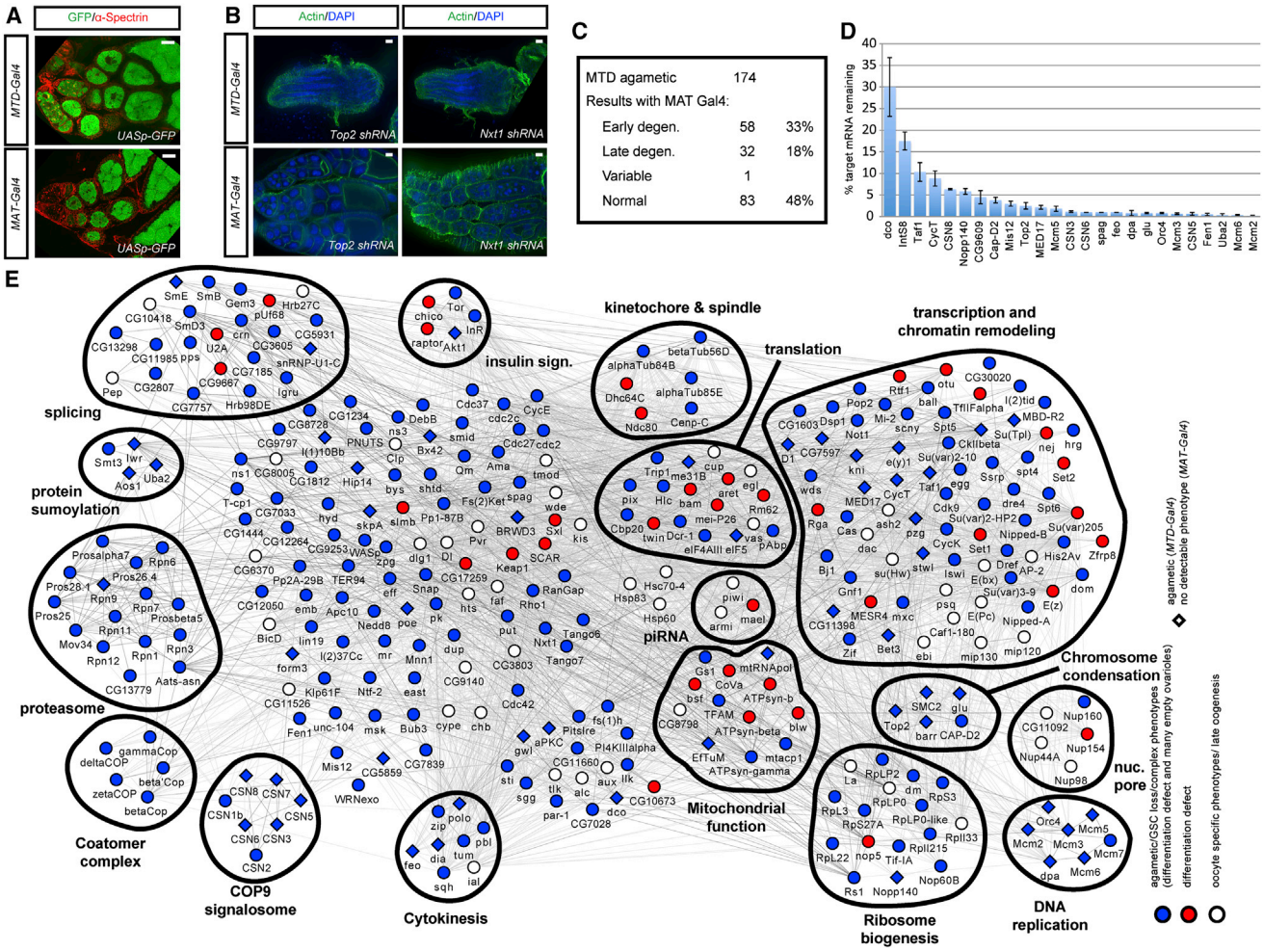


Figure 2. Quality Control and Regulatory Network for Genes Identified from the Screen

(A) Expression patterns of *UASp-GFP* using the *MTD-Gal4* or *MAT-Gal4* drivers.

(B) Examples of the MAT screen. *Top2* or *Nxt1* shRNA is induced by *MTD-Gal4* or *MAT-Gal4*, and the ovaries are stained for actin and DAPI. Both shRNAs result in agametic phenotypes with *MTD-Gal4*, but *Top2* shRNA produces normal eggs with *MAT-Gal4*, whereas *Nxt1* is still defective with *MAT-Gal4*.

(C) Summary of the secondary *MAT-Gal4* screen of 174 agametic lines. Of the RNAi lines, 83 (48%) produce normal eggs with *MAT-Gal4*, indicating that these genes have GSC- or cystocyte-specific functions.

(D) qPCR experiments assessing the knockdown efficiency of a select set of shRNAs in 0–4 hr eggs laid from *MAT-Gal4/shRNA* females. A total of 25 genes are ranked according to the degree of knockdown. x axis, genes tested; y axis, percent (%) target mRNA remaining. Data are mean ± SEM.

(E) Network of genes identified by the germline screen. Genes are shown as nodes, and the node color and shape indicate the observed phenotype in the screen. Red circle indicates differentiation defect, blue circle shows agametic/stem cell loss, white circle indicates other oogenesis defects, and blue diamond shows agametic (MAT normal). Overall, red genes represent those required for GSC differentiation, and blue ones are those required for GSC maintenance.

The edges denote the interactions of the genes and are represented in different gray tones: light edges are text mining/genetic interaction based, and dark edges represent protein-protein interaction data.

Scale bars, 20 μm. See also Figure S2.

arguing that it has GSC-specific functions (Figure 2B). Therefore, this secondary screen allows us to identify genes specific for a function in the germarium, including GSCs (Figure 2C; Table S1).

To test the efficiency of RNA knockdown, we selected a set of shRNAs for quantitative RT-PCR analysis and antibody staining. For qPCR, we chose 25 shRNA lines that are agametic with *MTD-Gal4* but produce normal eggs with *MAT-Gal4*. We prepared RNA from 0–4 hr eggs laid from *MAT-Gal4/shRNA* females, where most of the mRNAs are deposited during oogenesis. Compared to control shRNA, RNA levels for the target gene are reduced to <10% in 22 of the 25 lines (Figure 2D), suggesting that the shRNAs are highly efficient in knocking down gene expressed during oogenesis. Furthermore, we evaluated the knockdown efficiency at the protein level using antibodies, such as Akt1, Hts, HP1, Osa, and Brm (Brahma) (see below). For example, expression of *Akt1 shRNA* with *MAT-Gal4* produces normal eggs, although its protein level is significantly reduced in the germline cells (Figure S2A). Furthermore, *hts shRNA* induces an almost complete depletion of the fusome and ring canal-specific forms of the Hts protein in their respective region when expressed *by MTD-Gal4* or *MAT-Gal4*

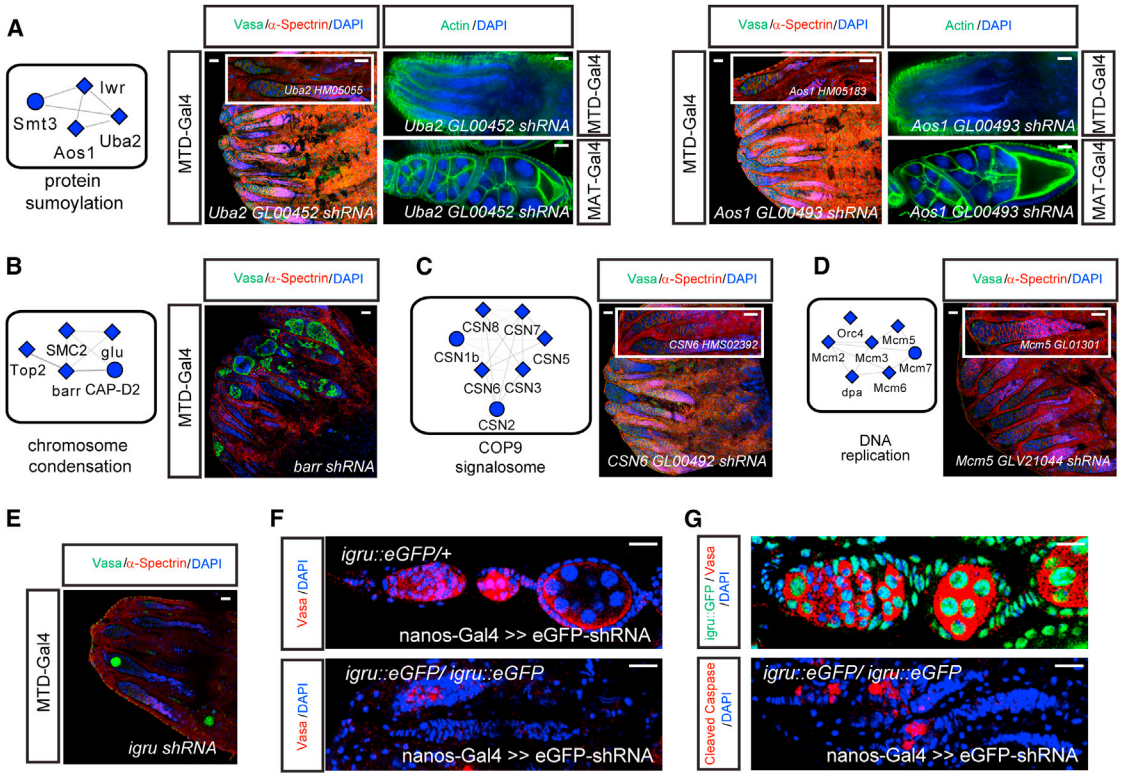


Figure 3. Genes and Complexes Required for GSC Maintenance

(A–D) Identified protein complexes required for GSC maintenance. Complexes are enlarged from Figure 2E. RNAi against a representative gene from each complex is shown, and other genes in the complex have a similar phenotype when knocked down by RNAi. (A) Knockdown of Uba2 or Aos1 by two independent RNAi constructs using *MTD-Gal4* or *UAS-dcr2; nanos-Gal4* results in a depletion of the germline. *MAT-Gal4*-mediated knockdown of these genes does not induce an obvious phenotype at later stages of oogenesis. (B–D) Knockdown of members of the respective complex results in a depletion of the germline. Independent shRNA constructs are shown in insets.

(E) Knockdown of *igru* (CG11266) with *MTD-Gal4* results in a depletion of the germline.

(F) Trap-mediated loss of function validates the loss of Vasa-positive germline cells upon loss of *igru* function.

(G) *igru::GFP* is expressed throughout the germline, including high-level expression in GSCs and low expression levels in the cystocyte region (top panel). Upon loss of igru function, remaining germline cells stain positive for Vasa and cleaved caspase 3 (lower panel).

Scale bars, 20 μm. See also Figure S3 and Table S2.

(Figure S2B). These proof-of-principle experiments show that the shRNA lines achieve efficient knockdown in our screen.

Gene Network Underlying GSC Self-Renewal

To better visualize our screening results, we generated a gene-interaction network querying publicly available databases containing protein-protein interactions, yeast two-hybrid interactions, genetic interactions, and text-mining data (Figure 2E). We divided the phenotypes into different categories including differentiation defects, GSC loss, late oogenesis, agametic, agametic (MAT normal), and agametic (MAT defective) and arranged them into functional groups (Figure 2E). To identify protein complexes in this network, we used COMPLEAT to perform a complex-enrichment analysis (Figure S3) (Vinayagam et al., 2013). This analysis allowed us to identify several protein complexes required for GSC maintenance, such as complexes involved in mRNA splicing, the COP9 signalosome (CSN), protein sumoylation, DNA replication, and condensin complexes. CSN is a highly conserved, eight-subunit protein complex that is involved in diverse cellular and developmental processes. The most studied CSN function is regulation of protein degradation, but recent data suggest that CSN also regulates transcription. RNAi targeting of *CSN1b* and *CSN2/alien* using *nanos-Gal4* generates an empty germarium phenotype. In addition, *CSN3*, *CSN5*, *CSN6*, *CSN7*, and *CSN8* shRNA resulted in a complete loss of all germline cells with *MTD-Gal4* (Figure 3C) but produced normal eggs with *MAT-Gal4*, suggesting that CSN function is only required early in the GSC lineage. Similarly, SUMO protein has been detected in the nuclei of the GSC and cystocytes, suggesting a role in GSC regulation (Hashiyama et al., 2009). Interestingly, knockdown of the two SUMO-activating enzymes Uba2 and Aos1 with two independent RNAi constructs using *MTD-Gal4* resulted in a loss of GSCs, whereas expression of these shRNAs with *MAT-Gal4* resulted in the production of normal eggs (Figure 3A). Consistently, knockdown of the SUMO protein Smt3, and SUMO-conjugating enzyme Lwr, led to agametic ovaries, arguing that sumoylation is required for GSC maintenance. Finally, seven components of a DNA replication complex (Mcm2, Mcm3, Mcm4/Dpa, Mcm5, Mcm6, Mcm7, and Orc4) and five components of the condensin complex (SMC2, Barr,

Glu, Top2, and CAP-D2) generated an agametic phenotype when knocked down by *MTD-Gal4*, and most of them produced normal eggs (despite signs of lower DNA content in nurse cells upon knockdown of Mcm-complex genes) with *MAT-Gal4* (Figures 3B, 3D, and S2C). Thus, both DNA replication and condensin complexes are predominantly required in the germarium, potentially reflecting their roles in actively dividing GSCs and cystocytes and, to a lesser extent, for endoreplication of nurse cells. Importantly, we have confirmed the knockdown efficiency of many of these shRNAs using qPCR (Figure 2D). Besides defined molecular complexes, we identified many other interesting genes required for GSC maintenance, including *phosphatidylinositol 4-kinase IIIα* (*PI4KIIIα*), *glutamine synthetase 1* (*GS1*), and *heterogeneous nuclear ribonucleoprotein at 98DE* (*Hrb98DE*) (Figure S4A).

Furthermore, our screen identified genes involved in GSC regulatory processes for which no phenotypic data are available to date. For example, knockdown of *CG11266* (referred to as *inselgruppe* [*igru*] hereafter; see Experimental Procedures for details), a proposed splicing factor, resulted in an almost complete depletion of germline cells (Figure 3E), suggesting a role for *igru* in GSC maintenance. We selected this gene for independent validation experiments because a functional *igru::EGFP* trap line permits localization and high-stringency loss-of-function studies. We took advantage of the recently developed "trap-mediated loss-of-function" method that uses well-characterized EGFP shRNA lines to knock down EGFP-trapped genes (Neumüller et al., 2012). Consistent with the shRNA phenotype, we found that GFP-mediated knockdown of *igru* with two independent EGFP-specific shRNAs resulted in indistinguishable phenotypes, confirming the requirement of *igru* in GSC maintenance (Figure 3F; data not shown). The few remaining Vasa-positive germline cells strongly stained for cleaved caspase 3, suggesting a requirement for *igru* in germline cell survival (Figure 3G). Consistent with this role and the proposed molecular function of *igru*, we found strong nuclear *igru::EGFP* expression in GSCs and polyploid nurse cells. Lower levels of *igru* expression are conversely detectable in cystocytes (Figure 3G). Together, these results demonstrate that *igru* is required for GSC maintenance and suggest that our screen can phenotypically annotate previously unstudied genes.

Genes Important for Stem Cell Differentiation

RNAi knockdown of genes required for GSC or CB differentiation results in the accumulation of undifferentiated cells containing extra spectrosomes and/or fusomes. From our network analysis, we found several genes important for GSC differentiation, including components of the RNA-splicing machinery and mitochondrial ATP synthase complex. For example, three genes involved in RNA splicing were identified: *pUf68* (the homolog of human PUF60), *U2A*, and *CG9667* (the ISY1-splicing factor homolog) (Figure 4A). Although previous reports suggest that *pUf68* mutants display striking defects in the splicing of *otu*, they only show late oogenesis and egg morphology phenotypes (Van Buskirk and Schüpbach, 2002). The strong differentiation defects associated with *pUf68* knockdown (Figure 4A) resemble the strongest *otu* phenotype, potentially reflecting its role in *otu* splicing. Interestingly, knockdown of three genes (*blw*/ATPsyn-α, ATPsyn-β, and ATPsyn-b) encoding subunits of the ATP synthase complex, as well as *cytochrome c oxidase subunit Va* (*CoVa*), also caused differentiation defects (Figure 4B). These data suggest that mitochondrial dysfunction is associated with GSC differentiation defects. Other interesting genes involved in GSC differentiation include *Prpk*, homolog of P53-regulating kinase; *zfrp8* (phenotype confirmed by two independent shRNAs), zinc finger protein involved in *Drosophila* hematopoietic stem cell regulation (Minakhina and Steward, 2010); the transcription initiation factor *TfIIFα*, *SCAR*, WAVE/SCAR complex component; *slmb*, F box/WD40 E3 ubiquitin ligase; *keap1*, regulator of cellular redox state; *CG10426*, inositol polyphosphate-5-phosphatase; *Ndc80*, a kinetochore protein; *Dhc64c*, dynein heavy-chain 64C; *bsf*, a mRNA-binding protein; and *Ccn*, a potential growth factor (Figures 4C and S4B).

To study the underlying mechanisms, we selected four genes associated with strong differentiation defects, *U2A*, *Prpk*, *Zfrp8*, and *TFIIfα*. To characterize the loss-of-function phenotypes in greater detail, we stained these ovaries with available molecular markers Sxl, Bam, and Dad-LacZ. In wild-type (WT) germaria, the Sxl protein accumulates to high levels in GSCs and CBs, Bam is expressed in CBs and early cysts, and Dad-lacZ, a reporter of Dpp signal activation, is confined to the two to three GSCs (Figure 4D). Sxl accumulation is normal in *Prpk*, *Zrfp8*, and *TFIIfα shRNA* mutant ovaries; however, it is strongly reduced in *U2A* shRNA ovaries (Figure 4D). *U2A* encodes the *Drosophila* U2 snRNP, and the reduction of Sxl protein likely reflects its role in *sxl* splicing (Nagengast and Salz, 2001). Next, Bam protein expression is largely normal in *U2A* and *TFIIfα* shRNA ovaries, but reduced significantly in *Prpk* and *Zfrp8* shRNA ovaries, suggesting that these two genes control differentiation in a *bam*-dependent manner. To confirm these results, we overexpressed *bam* from a heat shock-inducible promoter in the *shRNA* knockdown background (Ohlstein and McKearin, 1997). Heat shock-induced *bam* (*hs-bam*) is able to rescue *Prpk* and *zfrp8 shRNA*, generating normally developed egg chambers and reversing the fertility phenotype (see Figure 4E for quantification and Figure S4C for representative images). On the other hand, *hs-bam* has no obvious effect in *U2A* and *TFIIfα shRNA* ovaries (Figures 4E and S4C). Finally, Dad-lacZ is expressed normally in *U2A*, *Prpk*, and *TFIIfα shRNA* ovaries but is ectopically induced in *Zfrp8* ovaries. These results indicate that *Zfrp8* regulates *bam* expression through controlling Dpp signaling. Together, we have identified factors that control multiple steps of GSC differentiation.

Transcriptional Network for GSC Regulation

Because stem cell self-renewal is controlled by key transcription factors and by epigenetic regulation, we decided to analyze in particular these two classes of genes. To systematically study transcriptional regulation in GSC self-renewal, we built a subnetwork containing 65 experimentally verified or computationally predicted transcription factors and chromatin regulators (Figure 5A). Among those, we identified several previously known GSC transcriptional regulators (Figure 5B). Twin, a subunit of the CCR4-NOT complex, is required for GSC differentiation (Morris et al., 2005). The ATP-dependent chromatin-remodeling factor ISWI (Xi and Xie, 2005), the DNA-associated protein Stonewall (Maines et al., 2007), the histone H3K9 trimethylase Egg (Wang et al., 2011), the histone H2B ubiquitin protease

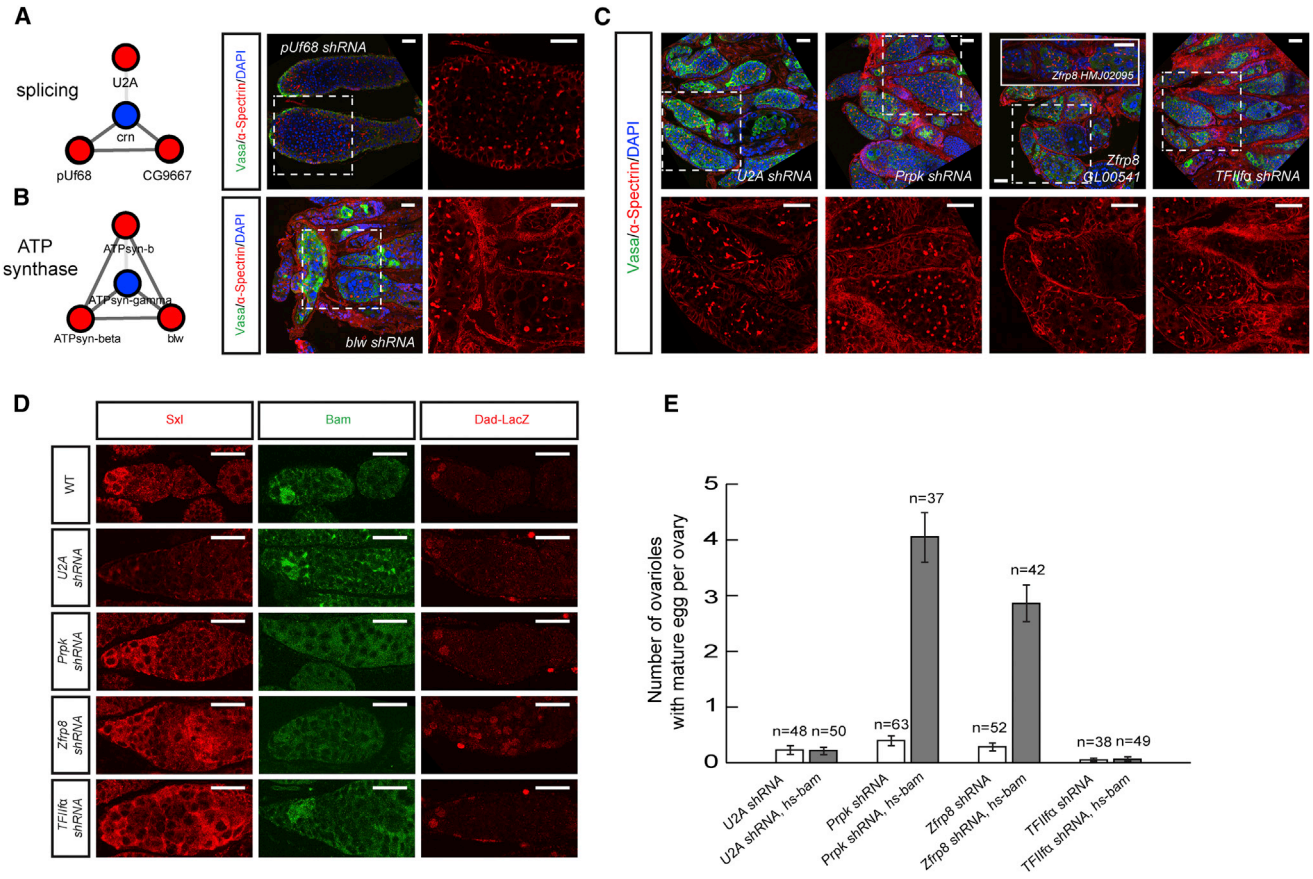


Figure 4. Genes and Complexes Required for GSC Differentiation

(A and B) Identified protein complexes required for GSC differentiation. RNAi against one representative gene from each complex is shown. Ovaries expressing RNAi targeting *pUf68* and *blw* are stained for α-Spectrin, Vasa, and DAPI. Areas marked by the dashed squares are enlarged.

(C) shRNAs against *U2A*, *Prpk*, *zfrp8*, *or TFIIfα* expressed by *MTD-Gal4* lead to strong differentiation defects. Ovaries are stained for α-Spectrin, Vasa, and DAPI, and *Zfrp8* phenotype is confirmed by two independent shRNAs.

(D) Ovaries expressing *U2A*, *Prpk*, *zfrp8*, *or TFIIfα* shRNAs are stained for Sxl, Bam, or Dad-lacZ. Sxl and Bam experiments were done using *MTD-Gal4*, and Dad-lacZ experiments were done using *nanos-Gal4*.

(E) Quantification of *hs-bam* rescue experiments. shRNAs against *U2A*, *Prpk*, *zfrp8*, *or TFIIfα* are expressed using *nanos-Gal4* with or without *hs-bam* expression. The number of ovarioles with mature egg per ovary is shown, and "n" is the number of ovaries examined. Data are mean ± SEM.

Scale bars, 20 μm. See also Figure S4C.

Scny (Buszczak et al., 2009), and the transcription elongation factor Spt6 (Neumüller et al., 2012) are essential for GSC maintenance. Besides these known factors, our screen identified several additional transcriptional regulators of GSC self-renewal. For example, knockdown of CCR4-NOT subunit Rga, Paf1 complex subunit Rtf1, *Drosophila* HP1/Su(var)205, Polycomb group protein E(z), histone acetyltransferase nej/dCBP, and histone H3K36 methylase Set2 is associated with defects in differentiation; whereas PIAS homolog Su(var)2-10 or components of the basic transcriptional machinery, including the TATA box-binding protein (TBP)-associated factor Taf1, e(y)1/TafII40, or the mediator component MED17, and the transcription elongation factor Spt4, Su(Tpl), are important for GSC maintenance (Figures 5B and S5A). As an example, we probed the *Su(var)205 shRNA* ovaries with HP1 antibody and the heterochromatic marker H3K9me3. As shown in Figures S5B and S5C, HP1 staining is abolished from these germline cells, and H3K9me3 staining is also reduced. These results suggest that the shRNA effectively knocks down Su(var)205 protein level, which is important to maintain heterochromatin structure in the germline. Together, these results provide a first step toward generating a complete transcription factor network regulating GSC self-renewal.

Next, we focused our attention on the ATP-dependent chromatin-remodeling factor Dom. Dom was reported not to be required in GSCs (Xi and Xie, 2005), but our data suggested that *dom* is potentially required for GSC maintenance because *dom shRNA* induced by *MTD-Gal4* generates a loss of stem cell phenotype (Figure 5B). To clarify whether *dom* is required for GSC maintenance and to confirm the specificity of the knockdown, we used the trap-mediated loss-of-function method. We used a homozygous-viable *dom-EGFP* trap line and found that Dom-EGFP is expressed ubiquitously in the germline (Figure 5C), consistent with a potential requirement in GSCs. Dom is a nuclear protein that presumably localizes to active sites of polymerase II (Pol II) transcription because we did not detect

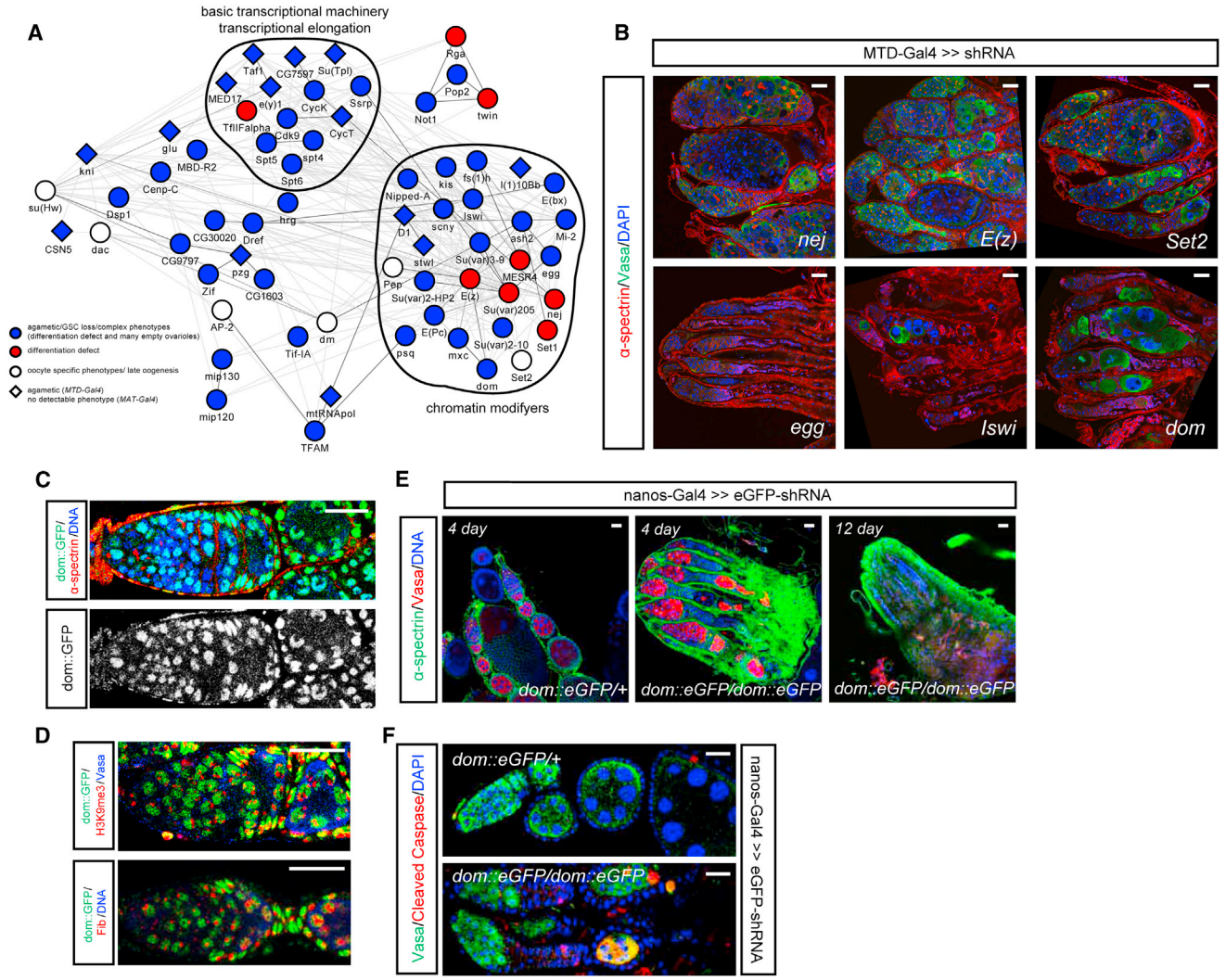


Figure 5. Transcription Factor Network Regulating GSC Self-Renewal

(A) Network for transcription factors and chromatin regulators identified from our screen. Nodes and edges are represented as in Figure 2E.

(B) shRNAs are expressed using *MTD-Gal4*, and ovaries are stained for α-Spectrin, Vasa, and DAPI. shRNAs against *nej*, E*(z)*, and *Set2* cause differentiation defects, whereas shRNAs against *egg*, *lswi*, and *dom* result in agametic or stem cell loss phenotype.

(C) *dom-EGFP* trap ovaries stained for α-Spectrin and DAPI show the expression pattern of Dom.

(D) *dom-EGFP* ovaries stained for Fibrillarin or H3K9me3.

(E) Ovaries expressing *EGFP shRNA* using *nanos-Gal4* in the *dom-EGFP* heterozygous or homozygous background are stained for α-Spectrin, Vasa, and DAPI. Days indicate time after eclosion. Germ cells are lost in *dom* loss of function after 12 days compared to WT (data not shown).

(F) Ovaries expressing *EGFP shRNA* using *nanos-Gal4* in the *dom-EGFP* heterozygous or homozygous background are stained for cleaved caspase, Vasa, and DAPI.

Scale bars, 20 μm. See also Figure S5.

Dom-EGFP in nuclear domains positive for the heterochromatic marker H3K9me3 and the nucleolar marker Fibrillarin (Figure 5D). Trap-mediated loss of *dom* function in GSC resulted in a complete loss of GSCs 12 days after eclosion (Figure 5E). Prior to the loss of all germline cells, we noticed a high number of Vasa and α-Spectrin double-positive cells throughout the ovarioles, suggesting a cystocyte differentiation defect. This phenotype was only transitory because increased numbers of apoptotic cells lead to a progressive loss of germline cells (Figure 5F). Taken together, our results demonstrate a requirement for *dom* in GSC self-renewal and also in cystocyte differentiation.

Comparison of Germline and Neural Stem Cell Self-Renewal

To understand if different stem cell systems use similar mechanisms to regulate self-renewal and differentiation, we compared the results of our screen with a previous analysis of neural stem cell self-renewal (Neumüller et al., 2011). GSCs are regulated through a niche-dependent mechanism, and Nbs control self-renewal through intrinsic asymmetric cell division. Because both stem cell types are mitotically active, we expected basic cellular processes to be commonly required. However, cell-type-specific regulators

might be rare modulators of these commonly required cellular networks.

Importantly, the genes we identified in our GSC screen are similarly enriched in ovaries and the larval CNS (Figure 1F). Of the 366 genes identified in the GSC screen, 103 genes were reported to be required for Nb self-renewal (Figure 6B). A Gene Ontology (GO) term analysis for these overlapping genes suggests that GSCs and Nbs share a requirement for many cellular processes in the regulation of self-renewal (Figure 6A). In addition, we compared our gene-interaction network (Figure 2E) with the gene network for Nb self-renewal (Neumüller et al., 2011). From these analyses, we found common as well as distinct regulators in these two cell types. Importantly, many basic cellular processes such as DNA replication, cell division, histone modification, and splicing are commonly required in Nbs and GSCs. Additionally, many transcription factors, chromatin-remodeling genes, the proteasome, as well as ribosome genes are associated with stem cell self-renewal defects in both systems. To gain further insight into the extent of overlap between genes regulating stem cell maintenance in these two systems, we performed gene set enrichment analyses. We used previously published data for nucleolar size regulation (Neumüller et al., 2013), rRNA processing (Tafforeau et al., 2013), and cell division (Hayles et al., 2013; Kittler et al., 2007) from a diverse set of species and found these genes commonly enriched in both GSC and Nb maintenance gene sets. Conversely, these genes were not significantly enriched in the gene sets associated with GSC differentiation or late oogenesis defects (Figure 6C). These data suggest a common requirement for cell growth and cell division for maintenance of Nbs and GSCs.

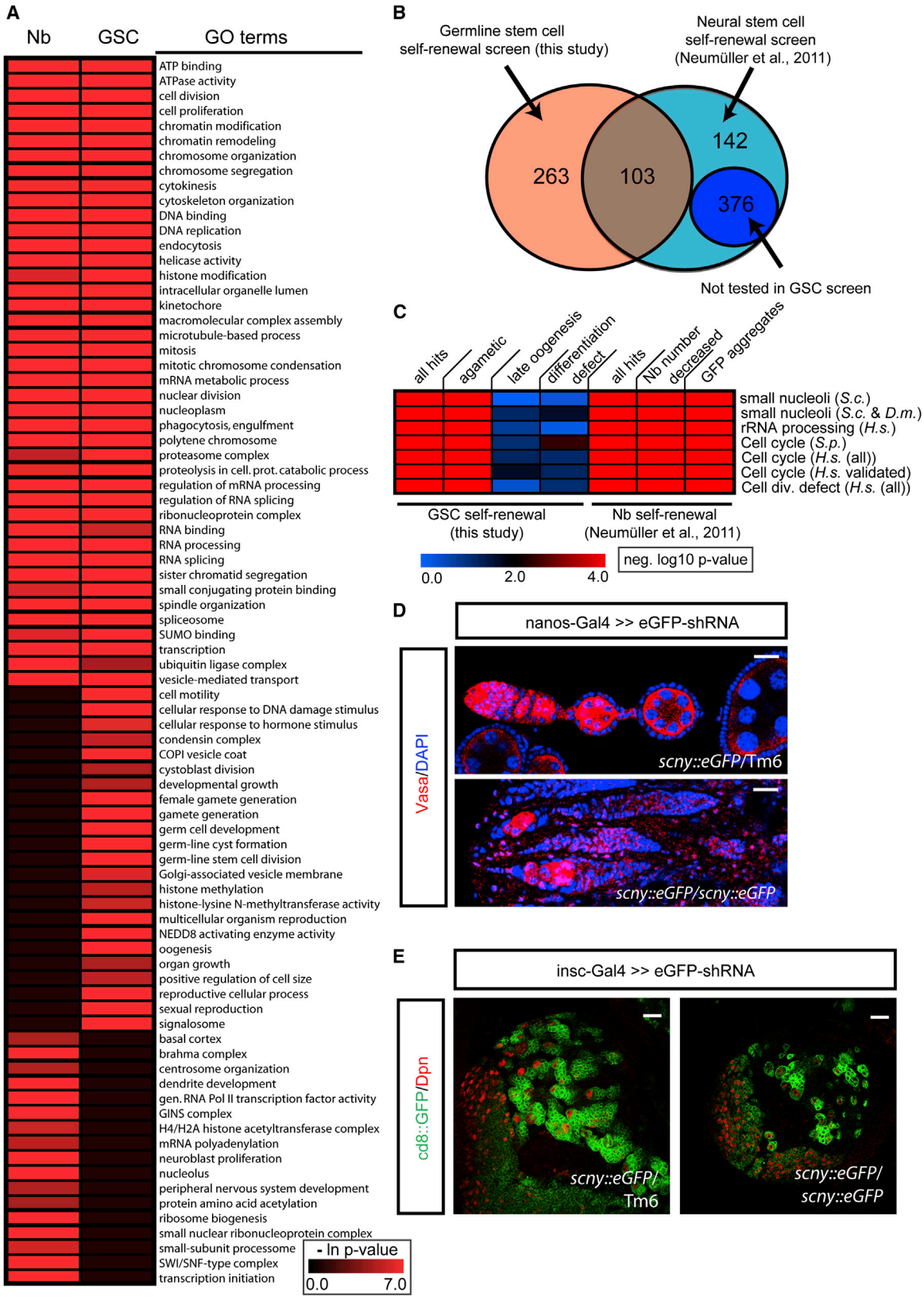
Genes only required in GSC regulation, but not in Nbs, include the COP9 signalosome complex, the protein sumoylation complex, several mitochondrial genes, and histone methyltransferases. It will be interesting to further study why these protein complexes are preferentially required in GSCs, but not Nbs. Conversely, knockdown of *brm*, *osa*, or *moira* is associated with an expansion of stem cell-like cells in type II Nb lineages (Neumüller et al., 2011). *osa* and *brm* are expressed at all stages in the GSC lineage. When expressed from *MTD-Gal4*, *osa* as well as *brm* shRNA constructs effectively deplete the respective proteins without inducing detectable phenotypes (Figures S6A and S6B). We next searched among the stem cell differentiation factors in GSC and Nb lineages for overlap. Interestingly, our analysis revealed an almost mutually exclusive set of differentiation factors (Ccn is the only factor shared between Nbs and GSCs), providing a comprehensive comparison of context-dependent differentiation and tumorigenesis in two stem cell lineages. However, because both studies (GSC as well as Nb self-renewal) do not cover the entire genome, we cannot formally exclude more potentially shared differentiation factors.

scny, encoding a ubiquitin-specific protease, was recently shown to be a common factor regulating self-renewal in germline, epithelial, and intestinal stem cell maintenance (Buszczak et al., 2009). However, *scny* has no described function in larval Nbs, raising the possibility that its function might be dispensable in this developmental cell type. To examine if *scny* is a general regulator of stem cell maintenance, we used trap-mediated loss of function to knock down *EGFP::scny* in a homozygous-viable and fertile EGFP trap line. *EGFP shRNA*-mediated knockdown in GSCs resulted in a depletion of Vasa-positive cells from the germline (Figures 6D and S6C), confirming previous results (Buszczak et al., 2009). Next, we studied *scny* function in Nbs by using a Nb-specific Gal4 line, *insc-Gal4* (Neumüller et al., 2011). Although EGFP shRNAs generated no phenotype in a heterozygous *scny-EGFP* background, they strongly reduced the number of Deadpan (Dpn)-positive Nbs in a homozygous *scny-EGFP* background (Figure 6E). We did not detect evidence for increased cell death upon *scny* loss of function in Nbs (data not shown). These data suggest that Scny is an essential stem cell maintenance factor in most if not all *Drosophila* stem cell types.

Set1 H3K4 Methyltransferase Is Required for Cystocyte, but Not Neuroblast, Differentiation

Methylation of histone 3 lysine 4 (H3K4) is a histone modification associated with active transcription. However, its function in adult stem cell regulation remains to be determined. In yeast, all mono-, di-, and trimethylation of H3K4 is catalyzed by a single Set1 enzyme, whereas in *Drosophila*, there are four known H3K4 methyltransferases: Trx, Ash1, Trr, and Set1 (Hallson et al., 2012). *Drosophila Set1* is located in the centric heterochromatin region and has been difficult to characterize by traditional genetic methods (Ardehali et al., 2011). Our systematic comparison of Nb and GSC self-renewal suggested that Set1 might be a GSC-specific self-renewal factor. *Set1 shRNA* expressed by *MTD-Gal4* leads to the co-occurrence of pseudoegg chambers filled with fusome- and spectrosome-containing cells and pseudoegg chambers containing >15 nurse cells as well as empty ovarioles, suggesting a role for Set1 in stem cell maintenance (as recently reported by Xuan et al., 2013) as well as differentiation (Figures 7A–7C). This result was confirmed using two additional independent shRNA lines (Figures 7A and S7C), indicating that Set1 is required during multiple processes in oogenesis. To show that the ectopic undifferentiated cells in *Set1 shRNA* ovaries retained their proliferative potential, we stained for the mitotic marker phosphorylated histone H3 (pH3). In WT ovaries, pH3-positive cells were restricted to the anterior tip of the germarium but were detected throughout *Set1 shRNA* ovaries (Figure S7B). Next, we found that *hs-bam* failed to fully rescue the *Set1 shRNA* phenotype (Figure 7D), suggesting that Set1 likely regulates GSC differentiation independent of Bam. Furthermore, we used an antibody against Mei-P26, which is expressed at low levels in GSCs, upregulated in cystocytes, and absent afterward (Liu et al., 2009; Neumüller et al., 2008). Compared to WT, *Set1 shRNA* ovaries have strong Mei-P26 staining throughout pseudo-egg chambers, indicating that the germline development is blocked at the cystocyte stage (Figure 7E).

Because Set1 is a major H3K4 methyltransferase, we determined its in vivo function using antibodies specific to different forms of methylated H3K4. In WT ovaries, all three modifications, mono-, di-, and trimethyl H3K4, were detected both in germaria and egg chambers (Figures 7F and S7D). *Set1 shRNA* had no effect on the pattern of H3K4me1 but almost completely abolished H3K4me2 and H4K4me3 staining in the germline, suggesting that Set1 acts as a major H3K4 di- and trimethyltransferase in the germline (Figures 7F, S7D, and S7E). Consistently, by using chromatin immunoprecipitation (ChIP) from



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fluorescence-activated cell sorting (FACS)-purified GSCs, we found an increased occurrence of trimethylated H3K4 on genomic regions covering genes that our screen identified as being associated with GSC maintenance or differentiation defects. Conversely, H3K4me3 levels are lower over genomic regions covering genes that did not result in a phenotype upon loss of function (Figure 7G). Importantly, a similar result was obtained in ChIP experiments for Pol II suggesting that H3K4me3 is predominantly associated with actively transcribed genes that are functionally required in GSCs (Figure 7H). Because we found H3K4me3 at genes regulating both GSC maintenance and differentiation as well as genes that control both processes, such as *Mei-P26* (Li et al., 2012) (Figure S7F), we postulate that altered expression of a yet to be determined set of key regulatory genes is underlying the observed phenotypes.

Because we have shown that Scny, which deubiquitylates histone H2B that is required for H3K4 methylation, is required for stem cell maintenance in both GSCs and Nbs, we tested whether Set1 controls self-renewal in Nb lineages as well. Surprisingly, knockdown of Set1 by *insc-Gal4* had no effect on Nb differentiation or maintenance (Figure 7I), although it reduced H3K4me3 to undetectable levels in those cells (Figure 7J). The neuronal markers Prospero (Pros) and Elav were expressed normally upon *Set1* knockdown, and Nb numbers as well as Dpn-positive progeny in type II lineages were similar to WT (Figure 7J; data not shown). These results indicate that unlike GSCs, neural stem cell differentiation and maintenance do not require Set1-mediated methylation of H3K4. It will be interesting to examine other stem cell systems and whether this mechanism applies to mammalian stem cells.

DISCUSSION

Most genes required for GSC self-renewal have previously been identified using homozygous mutant animals or mosaic analyses. Because of the limitations associated with both approaches (female sterile mutations usually represent hypomorphic alleles of zygotic lethals [Perrimon et al., 1986], and production of germline mosaics is relatively cumbersome [Perrimon et al., 1989, 1996], no large-scale screens have yet been performed to systematically identify genes involved in GSC self-renewal. Here, using transgenic RNAi in the *Drosophila* female germline, we screened 25% of the fly genome and identified 366 genes associated with specific stem cell defects. Based on our screen, we constructed a genetic network governing GSC self-renewal and identified several protein complexes essential for GSC regulation, such as the COP9 signalosome, protein sumoylation, the ATP synthase complex, as well as chromatin remodeling and transcription factors. This study significantly expands the factors known to be required for GSC self-renewal and will serve as a resource for future studies in GSC biology.

Detailed analysis of our data and a screen in Nbs allowed us to identify genes that control stem cell self-renewal in both systems. Our systematic analysis provides evidence that basic cellular processes as cell division, growth regulation, or splicing are commonly required in GSCs and Nbs. The molecular contexts in which these processes are embedded might however differ significantly. For example, alternative splicing has been shown to be a key regulatory step in both Nbs and GSCs. In Nbs, alternative splicing of the transcription factor *lola* has been suggested to be required for regulating self-renewal (Neumüller et al., 2011). GSCs conversely control this process through the alternative splicing of *sxl*, suggesting that basic cellular machineries are acting on different targets in GSCs and Nbs (Chau et al., 2009). We further evaluated the role of *dom* and *scny* in stem cell maintenance. Dom is an ATP-dependent chromatin-remodeling factor that has previously been implicated in somatic stem cell maintenance in the female ovary (Xi and Xie, 2005). A requirement in GSCs had not been documented potentially due to the use of hypomorphic alleles. Our analysis demonstrates a role of *dom* in GSCs. Because shRNA-mediated knockdown of *dom* in Nbs also induces stem cell loss (data not shown), we propose that *dom* is commonly required in different stem cells to control their maintenance. Similarly, the histone H2B deubiquitinase Scny has been suggested to be a general regulator of stem cell maintenance in adult stem cell lineages. We expand this function to Nbs and thus provide further evidence for a general role for histone deubiquitination in stem cell maintenance in *Drosophila*. Consistently, H2B monoubiquitination has been shown to significantly increase upon differentiation of human mesenchymal stem cells (Karpiuk et al., 2012). Altogether, these data establish histone ubiquitination as a common regulatory mechanism in stem cell biology.

Consistent with previous studies that found Nb and GSC daughter cell differentiation to be controlled by different mechanisms (intrinsic versus extrinsic asymmetric cell division), we did not obtain evidence for extensive overlap in genes controlling differentiation with our systematic approach. For example, loss of the *brm* complex is associated with tumor formation in type II Nb lineages and differentiation defects in intestinal stem cells (Jin et al., 2013; Zeng et al., 2013). Conversely, in the germline, loss of *brm* complex members is not associated with differentiation defects. These observations are in agreement with data on

Figure 6. Comparison of GSC and the Neural Stem Cell RNAi Screens

(A) Heatmap displaying overrepresentation of selected GO terms associated with genes identified in the GSC and the Nb screen.

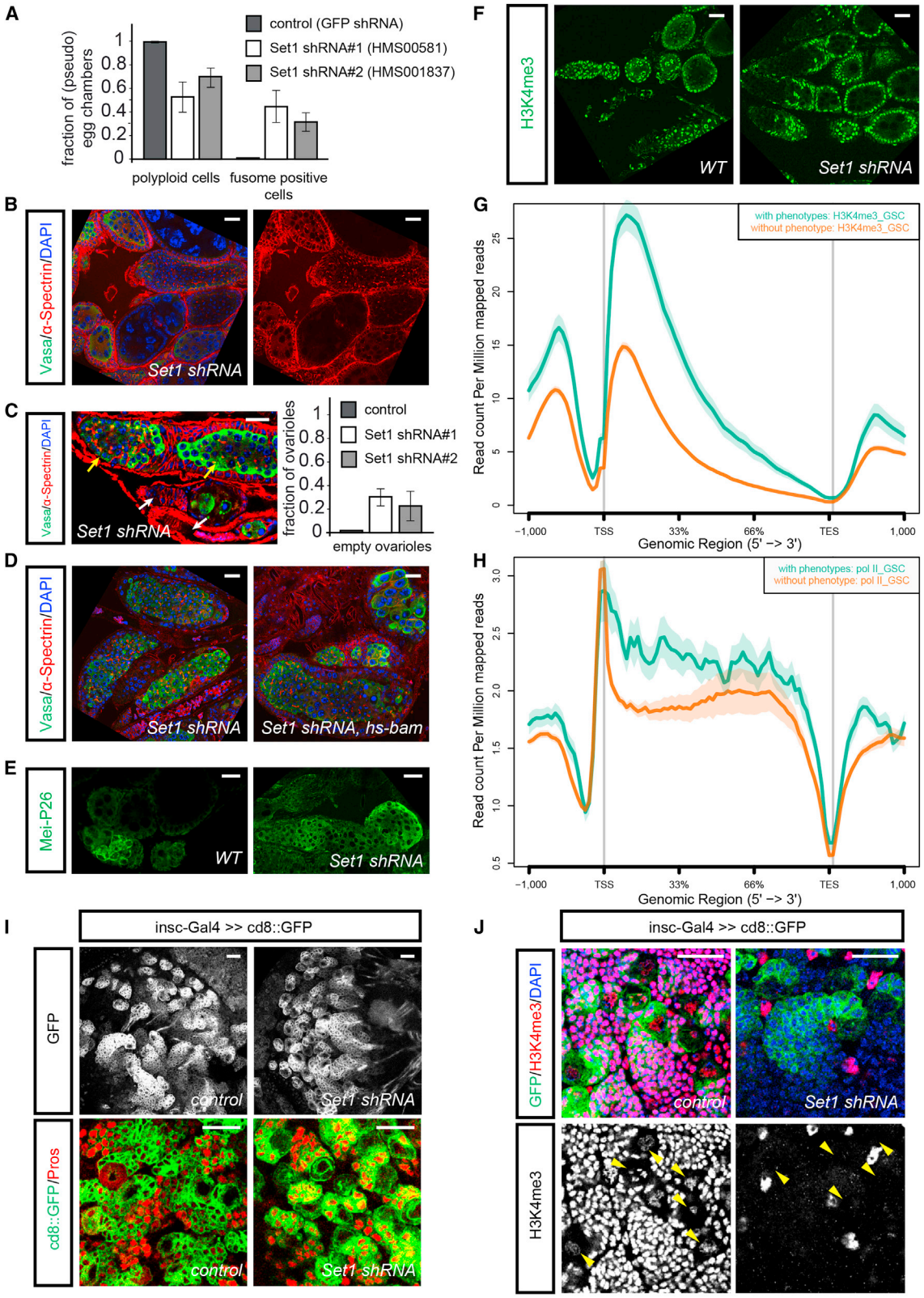
(B) Number of genes identified in the GSC and Nb screen. A total of 103 genes were found in both screens. Note that 375 identified genes in Nb screen were not tested in the germline screen.

(C) Comparative gene set enrichment between GSCs and Nbs of genes associated with small nucleoli, rRNA-processing defects, and cell division defects in the respective phenotypic categories.

(D) Ovaries expressing *EGFP shRNA* using *nanos-Gal4* in the protein trap *scny-EGFP* heterozygous or homozygous background stained for Vasa and DAPI.

(E) Larval brains expressing *EGFP shRNA* using *insc-Gal4* ≫ *CD8::GFP* in the *scny-EGFP* heterozygous or homozygous background stained for Nb marker Dpn (note that the EGFP shRNA does not target CD8::GFP).

Scale bars, 20 μm. See also Table S1 and Figure S6C.



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mutations in the SWI/SNF complex in different human cancers ranging from 0% to 75% in frequency (Shain and Pollack, 2013), underlining the value of *Drosophila* as a model system to study context-dependent tumorigenesis. Similarly, the gene *barricade* (*barc*) has been shown to result in an increased number of intermediate neural progenitor cells upon knockdown in Nb lineages (Neumüller et al., 2011), whereas depletion of *barc* results in a loss of germline cells (Figure S6D). These data suggest fundamental differences in lineage-specific tumorigenesis and suggest that an almost mutually exclusive set of differentiation genes operates in these two stem cell lineages. Interestingly, germline-specific genes can be ectopically expressed in certain brain tumor mutants and functionally sustain tumor growth (Janic et al., 2010). This ectopic expression might contribute to sustained tumor growth, and it will be interesting to determine if genes required for tumor maintenance are shared between Nb and GSC tumors. Together, our data provide systematic evidence that Nb and GSC lineages share an extensive stem cell maintenance network, whereas genetic programs regulating differentiation differ between these two cell types.

Importantly, we were able to identify several candidate genes with a specific requirement in GSCs. Our data demonstrate that Set1 and histone H3K4 trimethylation are important for germline differentiation and GSC maintenance but appear not to be required for Nb self-renewal. Xuan et al. recently documented a similar requirement of Set1 in GSC maintenance (Xuan et al., 2013). Our study, using multiple independent shRNA constructs, suggests that Set1 is required at multiple steps in the early GSC lineage, including cystocyte differentiation. Set1 is required for the bulk H3K4 trimethylation, a histone modification that has been associated with active sites of transcription. Consistent with the phenotypic spectrum of Set1 loss of function, we find H3K4me3 on genes that promote both GSC maintenance and differentiation. A recent report suggests that H3K4 methylation is dispensable for active transcription in somatic tissues (Hödl and Basler, 2012). Consistently, we find that in Nb lineages, key differentiation genes like Pros or Elav are normally induced and that H3K4me3 is not required for lineage progression. Conversely, our data suggest that H3K4 methylation is required for GSC self-renewal, and it will be interesting to determine if this differentiation defect is indeed linked to insufficient levels of active gene transcription of key differentiation genes.

EXPERIMENTAL PROCEDURES

RNAi Screen and *Drosophila* Strains

UAS-RNAi lines are generated by the TRiP and are available at the Bloomington Drosophila Stock Center (BDSC). For the RNAi experiments, we used a maternal triple-driver *MTD-Gal4* (BDSC 31777) or *UAS-dcr2; nanos-Gal4* (BDSC 25751) to drive expression of *UAS-RNAi* transgenes in GSCs, *MAT-Gal4* (BDSC 7063) for germline expression outside the germarium, and *insc-Gal4* for expression in larval Nbs. For trap-mediated loss-of-function analyses, we used *UAS-shRNAs* targeting EGFP as previously described (Neumüller et al., 2012). Protein trap lines scny::GFP, dom::GFP, and CG11266::GFP are described in Buszczak et al. (2007). We chose the name *inselgruppe* (German for "group of islands," abbreviated as *igru*) due to the few remaining, scattered Vasa-positive cells observed in the ovaries upon knockdown.

Immunofluorescence and Antibodies

Larval brains and female ovaries were stained as previously described (Neumüller et al., 2008). Briefly, tissues were dissected in PBS, fixed in 4% paraformaldehyde in PBST (PBS plus 0.1% Triton X-100). After blocking in 1% normal donkey serum in PBST for 1 hr, the samples were incubated with the primary antibody in the same solution at 4°C overnight. After three washes in PBST, samples were incubated with the secondary antibody for 2 hr at room temperature, washed in PBST for three times, and subsequently mounted in VECTASHIELD. The following antibodies were used: mouse anti-α-Spectrin (3A9; Developmental Studies Hybridoma Bank [DSHB]); rabbit anti-Vasa (Santa Cruz Biotechnology); mouse anti-Bam (DSHB); mouse anti-Sxl (M18; DSHB); mouse anti-LacZ (Promega); mouse anti-Osa (DSHB); guinea pig anti-Brm (gift from P. Harte); rabbit anti-Akt (Cell Signaling Technology); mouse anti-HP1 (C1A9; DSHB); rabbit anti-Mei-P26 (gift from P. Lasko); mouse anti-Hts (1B1; DSHB); mouse anti-Hts RC (DSHB); mouse anti-Pros (MR1A; DSHB); mouse anti-Elav (9F8A9; DSHB); guinea pig anti-Dpn (gift from J. Skeath); rabbit anti-phospho-Histone H3 (Millipore); rabbit anti-H3K4me3 (Cell Signal); mouse anti-H3K4me2 (Active Motif); rabbit anti-H3K4me1 (Active Motif); mouse anti-H3K9me3 (Abcam); mouse anti-Fibrillarin (Abcam); rabbit anti-GFP (Abcam); rabbit anti-cleaved caspase 3 (Cell Signal); Alexa 488-phalloidin (Molecular Probes); and DAPI (Molecular Probes). All images were taken on a Leica SP5 microscope.

qPCR

Total RNA was extracted from 0- to 4-hr-old eggs derived from *MAT-Gal4/shRNA* females using TRIzol (Invitrogen) and purified through RNeasy MinElute

Figure 7. Set1 Regulates GSC but Not Nb Self-Renewal

(A) Quantification of the *Set1* loss-of-function phenotype in the germline by *MTD-Gal4* (bars represent the mean ± SD of the observed frequencies; n = 175 pseudoegg chambers [HMS00581], n = 105 pseudoegg chambers [HMS001837]).

(B) Ovaries expressing *Set1 shRNA* (HMS00581) by *MTD-Gal4* are labeled by α-Spectrin, Vasa, and DAPI staining.

(C) Co-occurrence of pseudoegg chambers filled with undifferentiated fusome-containing cells (yellow arrows) and empty ovarioles (white arrows) in *MTD/Set1 shRNA* ovaries. Quantification of the empty ovariole phenotype (bars represent the mean ± SD of the observed frequencies; n = 74 ovarioles [HMS00581], n = 55 ovarioles [HMS001837]).

(D) Overexpressing *bam* using *hs-bam* fails to fully rescue the differentiation defects in *Set1 shRNA/nanos-Gal4* background, as shown by α-Spectrin, Vasa, and DAPI staining.

(E) Mei-P26 antibody staining in WT and *Set1 shRNA/MTD-Gal4* ovaries.

(F) H3K4me3 staining in WT and *Set1 shRNA/MTD-Gal4* ovaries.

(G) H3K4me3 ChIP from FACS-purified GSCs showing increased levels of lysine4 trimethylation at genes with a phenotype (green) in our screen over genes without a detectable phenotype (brown). x axis depicts 1,000 bp upstream of the transcriptional start site (TSS), the length of the gene bodies in percentage, the transcriptional end site (TES), and 1,000 bp downstream.

(H) Pol II ChIP from FACS-purified GSCs showing an increased association of Pol II at genes with a phenotype (green) in our screen over genes without a detectable phenotype (brown). x axis depicts 1,000 bp upstream of the transcriptional start site, the length of the gene bodies in percentage, the transcriptional end site, and 1,000 bp downstream.

(I and J) Larval brains expressing *Set1 shRNA* or no RNAi (control) using *insc-Gal4 ≫ CD8::GFP* stained by neuronal marker Pros (I) or H3K4me3 and DAPI (J). Yellow arrowheads point to Nbs.

Scale bars, 20 μm. See also Figure S7.

Cleanup Kit (QIAGEN). cDNA was generated from 1 μg of purified RNA using iScript cDNA Synthesis Kit (Bio-Rad). qPCR analysis was performed twice with technical triplicates in iQ SYBR Green Supermix (Bio-Rad), using a CFX96 real-time PCR detection system (Bio-Rad). Query transcript detection was normalized to the expression of three reference genes: *α-tubulin*, *rp49*, and *nuclear fallout*. Fold change was calculated in comparison to an shRNA knockdown targeting the *white* gene. Primers are selected using FlyPrimerBank.

FACS Isolation and ChIP

GFP-positive GSCs and CBs are isolated from the ovaries of *vasa-GFP/+; nos-gal4/UASp-tkv^{CA}* and *vasa-GFP/+; bam^{Δ86}/bam^{Δ86}* using FACS according to the previously published procedure by Song et al. (2004). The ChIP experiments were performed based on the published protocol by Zeitlinger et al. (2007), and the antibodies used are H3K4me3 (Abcam; ab8580) and Pol II (Abcam; ab5131). Bioinformatics analyses are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables, and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.01.020.

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