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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Protein synthesis and degradation are essential to regulate germline stem cell homeostasis in *Drosophila* testes

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ABSTRACT

The homeostasis of self-renewal and differentiation in stem cells is controlled by intrinsic signals and their niche. We conducted a large-scale RNA interference (RNAi) screen in *Drosophila* testes and identified 221 genes required for germline stem cell (GSC) maintenance or differentiation. Knockdown of these genes in transit-amplifying spermatogonia and cyst cells further revealed various phenotypes. Complex analysis uncovered that many of the identified genes are involved in key steps of protein synthesis and degradation. A group of genes that are required for mRNA splicing and protein translation contributes to both GSC self-renewal and early germ cell differentiation. Loss of genes in the protein degradation pathway in cyst cells leads to testis tumors consisting of overproliferated germ cells. Importantly, in the Cullin 4-RING E3 ubiquitin ligase (CRL4) complex, we identified multiple proteins that are crucial to GSC self-renewal: pic/DDB1, a CRL4 linker protein, is not only required for GSC self-renewal in flies but also for maintenance of spermatogonial stem cells (SSCs) in mice.

KEY WORDS: *Drosophila*, Testis, Germline stem cells, Differentiation, Self-renewal

INTRODUCTION

Stem cells are required for development and tissue homeostasis because they are capable of self-renewal and differentiation. They are maintained by a microenvironment that has been described as the stem cell niche. In the *Drosophila* testis, two populations of stem cells – germline stem cells (GSCs) and cyst stem cells (CySCs) – are maintained through their interactions with hub cells at the apical tip of the testis (de Cuevas and Matunis, 2011; Spradling et al., 2011). A male GSC divides asymmetrically to generate one daughter that attaches to the hub cells and maintains its 'stemness', and another called a gonialblast (GB) that exits the niche and begins the process of differentiation. GBs commonly go through several rounds of mitotic division to form transit-amplifying (TA) spermatogonia before switching to meiosis and finally forming mature sperm cells (White-Cooper, 2009). CySCs self-renew and give rise to daughters that differentiate into somatic cyst cells. Two cyst cells surround the progenies of one GB and form a cyst, and cyst cells codifferentiate with the germ cells they enclose (de Cuevas and Matunis, 2011).

Hub cells secrete Unpaired (Upd), a ligand that activates the Janus kinase signal transducer and activator transcription (JAK-STAT) pathway in both GSCs and CySCs (Kiger et al., 2001; Tulina and Matunis, 2001). Activation of this pathway is required for maintaining the attachment of GSCs to the hub and for the self-renewal of CySCs. In addition to Upd, Hedgehog (Hh), which is produced in hub cells and activates the Hh signaling pathway in CySCs, regulates CySC number and maintains their pluripotency (Michel et al., 2012; Zhang et al., 2013). Early germ cells express the epidermal growth factor receptor (EGFR) ligand Spitz, which activates the EGFR in adjacent cyst cells (Schulz et al., 2002). EGFR stimulation, in turn, leads to a rearrangement of the cytoskeleton in cyst cells that leads to envelopment of the GBs and activates a mitogen-activated protein kinase (MAPK)-dependent cascade that promotes differentiation of the enclosed germ cells (Kiger et al., 2000; Tran et al., 2000). Decapentaplegic (Dpp) and Glass bottom boat (Gbb), which are two BMP-like molecules secreted from somatic cells, block the expression of Bag of marbles (Bam), a key differentiation factor in GSCs and early germ cells (Kawase et al., 2004). During TA cell divisions, the initiation of Bam expression terminates mitotic divisions in the spermatogonia and triggers spermatocyte development (Gonczy et al., 1997). In addition to the key molecules in the signaling pathways mentioned above, many other factors also regulate the self-renewal and differentiation of stem cells in the testis. Epigenetic factors, microRNAs, actin-binding proteins, nuclear lamins and RNA-binding proteins have all been shown to affect the activity of stem cells in the testis (Chen et al., 2014, 2013; Cherry and Matunis, 2010; Eun et al., 2013; Pek et al., 2009; Shields et al., 2014). Traditional studies of genes that regulate stem cell activity in testes have relied on mosaic analysis or genetic screens for male sterility in homozygous mutant animals. Although many regulators of GSC self-renewal and differentiation have been identified, systematic screens have yet to be conducted. Recently, a large-scale *in vivo* RNAi screen in female fly GSCs identified a regulatory network of GSC self-renewal and differentiation (Yan et al., 2014). GSCs in the *Drosophila* testis share many features with female GSCs in the ovary. Therefore, data from female GSCs might also inform our understanding of GSCs in the testis. Comparisons of these systems have revealed many sex-specific differences between testes and ovaries. Therefore, a systematic analysis of GSC activity in the testis will provide further insights into the regulatory networks of GSCs.

In this study, we systematically analyzed GSC self-renewal in the testis by using RNAi *in vivo* (Fig. 1A). We screened 2881 RNAi lines corresponding to 2937 genes and identified 221 genes that are required for GSC self-renewal and differentiation. We further

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analyzed the functions of these genes in TA spermatogonia and cyst cells. We carried out complex analysis and discovered that many genes involved in protein synthesis and degradation are required for homeostasis of GSCs and their niches in the testis. In addition to the many gene products previously identified to either promote GSC self-renewal or trigger early germ cell differentiation, we uncovered

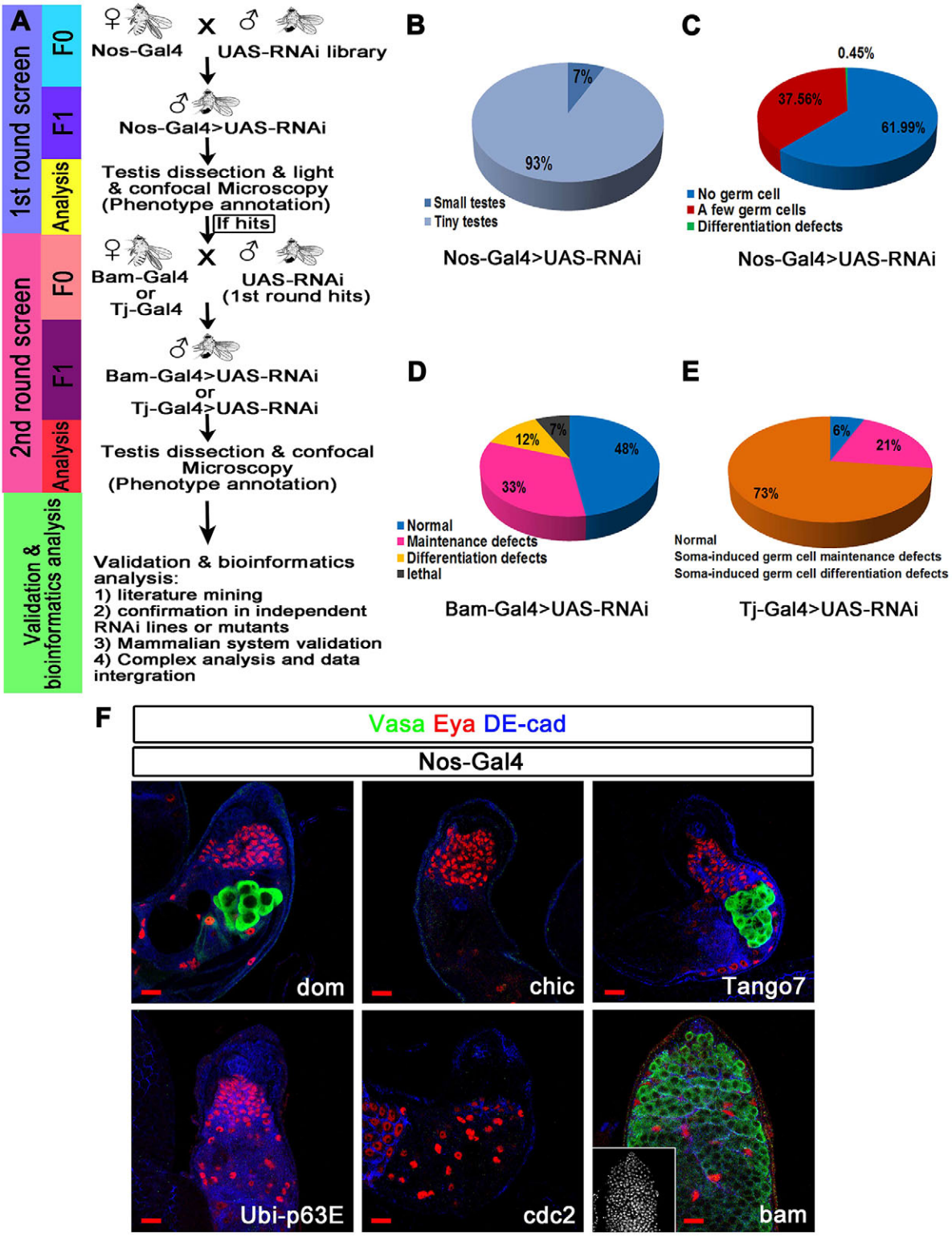

Fig. 1. Large-scale transgenic RNAi screen and quality control. (A) Transgenic RNAi screening strategies in the fly testis. (B-F) Phenotype annotations of hits. Phenotypes of the testes were divided into several categories and the proportions of each category are shown in pie charts. Phenotype annotations for the *nos-Gal4* screen (B,C), the *bam-Gal4* screen (D) and the *tj-Gal4* screen (E). (F) Typical phenotypes of testes with RNAi indicated genes in germ cells. These genes were reported to be necessary for testis stem cell self-renewal and differentiation. Anti-Vasa (green), anti-Eya (red) and anti-DE-cad (blue) label germ cells, cyst cells and hub cells, respectively. The inset shows Hoechst 33342 staining. Scale bars: 20 µm.

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a group of genes that are required for both processes. We also demonstrate that CRL4 complexes play crucial roles in GSC self-renewal in both fly and mouse testes. Our study is unique in that it not only serves as a rich source of information to study GSCs and their niche in flies, but also sheds light on the regulatory networks of stem cells in mammalian testes.

RESULTS

GSC self-renewal screen in fly testes

To systematically identify genes essential for GSC self-renewal and differentiation, we conducted a large-scale RNAi screen in the fly testis. We knocked down gene expression specifically in early germ cells by expressing UAS-shRNAs driven by *nos-Gal4* (Fig. S1A,A′). Because the majority of cells in the fly testis are germ cells, misregulation of their development can cause morphological collapse and lead to a small testis phenotype. We dissected adult male flies and isolated alleles associated with aberrant shape or size of the testis by light microscopy. To further analyze the phenotype, we immunostained the isolated alleles with markers for germ cells, cyst cells and hub cells.

In total, we screened 2881 RNAi lines from the TRiP (Transgenic RNAi Project) collection [Table S1 (https://dx.doi.org/10.6084/m9.figshare.3492410.v1) and Table 1], which corresponded to 2937 individual genes. We found that 233 lines targeting 221 genes caused the testis to become aberrant in shape and size [Table S2 (https://dx.doi.org/10.6084/m9.figshare.3492410.v1)]. Of these, knockdown of 93% and 7% of the genes led to tiny [testis length <50% of wild-type (WT) length] and small (testis length between 50% and 75% of WT length) testes, respectively (Fig. 1B). For example, knockdown of *eIF5* resulted in a small testis (Fig. 2C), whereas knockdown of *Cleavage and polyadenylation specificity factor 100* (*Cpsf100*) led to a tiny testis (Fig. 2E). On the basis of immunostaining data, we further divided the phenotypes into several categories (Fig. 1C): no germ cells present (61.99%) (Fig. 2F), a few germ cells present (37.56%) (Fig. 2D) and undifferentiated GSC-like cells accumulated in the absence of late-stage germ cells (0.45%) (Fig. 1F, bam).

Several genes necessary for stem cell self-renewal or differentiation were also identified in our screen, suggesting that our screening strategy is reliable (Fig. 1F). *dom* is essential for stem cell maintenance in the testis (Morillo Prado et al., 2013). *chic*, the *Drosophila* gene that encodes profilin, is required to maintain GSCs in a cell-autonomous manner, possibly by regulating the GSC-hub cell interface (Shields et al., 2014). In our screen, knockdown of *dom* or *chic* (Fig. 1F) with *nos-Gal4* led to a small testis with very few remaining germ cells, suggesting that *dom* and *chic* are indeed required for GSC self-renewal in the testis. Knockdown of *Tango7*, *Ubi-p63E* and *cdc2* (Fig. 1F), which have been implicated in spermatogenesis (D'Brot et al., 2013; Jin et al., 2005; Lu et al., 2013), led to a complete loss of germ cells. Interestingly, in testes with few or no germ cells, cyst cells were observed to expand (Fig. 1F, Eya staining) – a phenotype that has been reported in agametic testes (Gonczy and DiNardo, 1996). Our screen also identified Bam, a key regulator of GSC differentiation; consistent with the known functions of Bam, *nos>bam-RNAi* flies had small testes filled with undifferentiated germ cells (Fig. 1F, bam).

Several RNAi screens that have used the same collection of RNAi lines suggest that the off-target rates of this collection are low (Ni et al., 2011; Yan et al., 2014; Zeng et al., 2015). Of the 211 genes identified here, 31 turned out to have two independent shRNAi lines. For 12 genes, two independent RNAi lines targeting a single gene produced progenies with similar phenotypes when they were crossed with *nos-Gal4* flies (Fig. S1E-P,E′-P′), again suggesting that our datasets are reliable.

Table 1. Summary of RNAi lines used in this study

Vector	Line[a]	Hits[b]	RNAi
VALIUM20	2860	225	shRNA
VALIUM21	2	0	shRNA
VALIUM22	19	8	shRNA
Total	2881	233	

[a]The number of RNAi lines that were generated with indicated vectors used in this study; [b]The number of positive hits with each RNAi line.

Secondary screens identify genes required in TA spermatogonia and cyst cells

The genes required for GSC self-renewal could be genes that are essential for cell survival in general. To distinguish whether a gene is specifically required in GSCs, we further analyzed the 221 hits from the primary screen with secondary assays. *Bam-Gal4* was mainly expressed in TA spermatogonia and *tj-Gal4* was expressed in CySCs and cyst cells (Fig. S1B-D,B′-D′). We used *bam-Gal4* and *tj-Gal4* to drive the expression of shRNAs that targeted the genes identified in the primary screen. The resulting adult males were dissected and the testes were stained with various markers.

Several phenotypes were observed when genes were knocked down in spermatogonia by using *bam-Gal4* (Fig. 1D). About 48% of the lines showed no obvious phenotype (Fig. 2G), indicating that the corresponding genes are not essential for cell survival. Roughly 33% of the lines showed some loss of germ cell cysts, with many empty spaces found in the adult testis (maintenance defects, Fig. 2H). About 12% of the lines also had testes full of small germ cells, resembling the testes from *bam* mutant males (differentiation defects, Fig. 2I,J). This third category of lines showed the expansion of the Hoechst staining area (Fig. 2K-N), reduced branching of 1B1 staining (Fig. 2O-R) and loss of late-stage germ cells in phase contrast microscopy analyses (Fig. 2S-V′). Expression of the differentiation factor Bam was completely abolished in the *bam* RNAi testis (Fig. 2P), suggesting that RNAi was effective. In other RNAi lines with differentiation defects (Fig. 2Q,R), *bam* expression persisted at low levels in over-proliferating cysts (Bunt and Hime, 2004; Kawase et al., 2004). This suggests that the corresponding genes for these 12% of lines are critical for the differentiation of germ cells. Finally, about 7% of the lines could not produce viable male progenies.

In the secondary screen using *tj-Gal4* (Fig. 1E), approximately 6% of the lines had grossly normal testes when genes were knocked down in cyst cells (Fig. 2W). About 21% of the lines showed some loss of the germ cell cyst, suggesting that the corresponding genes are required for cyst cells to maintain the germ cell population (soma-induced germ cell maintenance defects, Fig. 2X). Interestingly, about 73% of the lines accumulated undifferentiated germ cells and developed testis tumors (soma-induced germ cell differentiation defects, Fig. 2Y,Z), indicating that the corresponding genes in cyst cells are required for germ cell differentiation.

In total, 8 of 221 lines showed phenotypes only when their expression was driven by *nos-Gal4* but not when it was driven by *bam-Gal4* or *tj-Gal4*. This suggests that the corresponding genes of these lines are specifically required in GSCs but not in spermatogonia or cyst cells.

Interestingly, 27 lines produced progenies with few germ cells in testes when crossed with *nos-Gal4*, whereas they produced

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progenies with over-proliferated and undifferentiated early germ cells in testes when crossed with *bam-Gal4*. This suggests that the corresponding genes were required not only for GSC maintenance but also for early germ cell differentiation, two processes that are usually thought to be regulated by distinct factors.

Stem cell regulatory networks in testis

To understand the regulatory network of stem cells in the testis, we generated a gene-protein interaction network for the genes identified in this screen (Fig. S2). We queried available databases that contained information on protein-protein interactions, genetic

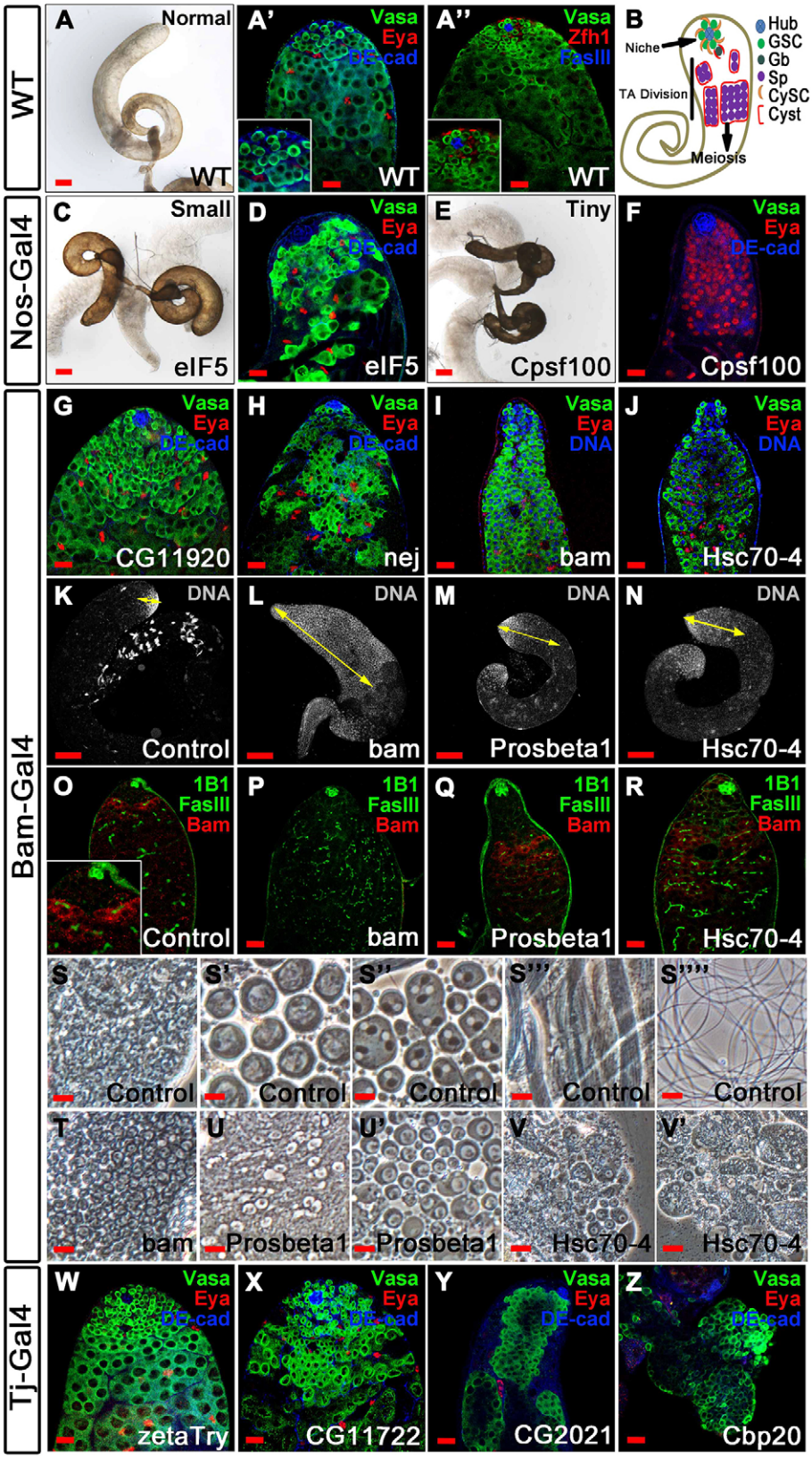

Fig. 2. Typical phenotypes observed in the testis RNAi screen. (A-A″) WT testes. Insets show the tip of the testis with hub cells, GSCs, and CySCs. (B) Diagram showing stem cells and early germ cells in *Drosophila* testis. (C-F) Knockdown of *eIF5* (C,D) and *Cpsf100* (E,F) with *nos-Gal4* resulted in small or tiny testes with few or no germ cells. (G-V′) Typical phenotypes observed in the *bam-Gal4* screen. (G) Knockdown of *CG11920* with *bam-Gal4* results in normal testes. (H) Knockdown of *nej* with *bam-Gal4* results in testes with some loss of germ cell cysts. (I,J) Knockdown of *bam* and *Hsc70-4* with *bam-Gal4* results in testes full of small undifferentiated germ cells. Hoechst staining regions are expanded in testes with *bam-Gal4*-driven *bam* (L), *Prosbeta1* (M) and *Hsc70-4* (N) RNAi expression. Yellow arrows represent regions of early undifferentiated cells. (O-R) Pointed fusomes (1B1 staining) are present in undifferentiated germ cells of *bam*, *Prosbeta1* and *Hsc70-4* RNAi testes. Bam expression is reduced to undetectable levels in *bam* RNAi testes. Although Bam expression levels are reduced in *Prosbeta1* and *Hsc70-4* RNAi testes, the distribution of expression is more widespread compared with the control. (S-V′) Phase-contrast analysis of WT or RNAi testes. Spermatogonia (S), spermatocytes (S′), round spermatids (S″), elongated spermatids (S‴) and mature sperm (S⁗) are shown in WT testes. Some early germ cells are present in *bam* (T), *Prosbeta1* (U and U′) and *Hsc70-4* (V and V′) RNAi testes. (W-Z) Typical phenotypes observed in the *tj-Gal4* screen. (W) Knockdown of *zetaTry* with *tj-Gal4* in cyst cells caused no obvious testes defects. (X) Knockdown of *CG11722* with *tj-Gal4* in cyst cells led to a loss of some germ cell cysts in testes. (Y,Z) Knockdown of *CG2021* (Y) and *Cbp20* (Z) with *tj-Gal4* caused a proliferation of GSC-like cells and testis tumor. In the case of *CG2021* RNAi testes, overproliferated cells grew inside the lumen. However, overproliferated germ cells were invasive and grew outside of the testis lumen in *Cbp20* RNAi testes. Germ cells are labeled with anti-Vasa; differentiated cyst cells are labelled with anti-Eya; CySCs are labeled with anti-Zfh-1, whereas Cyst cells and hub cells are labelled with anti-DE-cad; and hub cells, TA spermatogonia and fusomes are labeled with anti-FasIII, anti-Bam and anti-1B1, respectively. DNA is stained with Hoechst 33342. Scale bars: 100 µm for A, C, E and K-N; 20 µm for all other panels.

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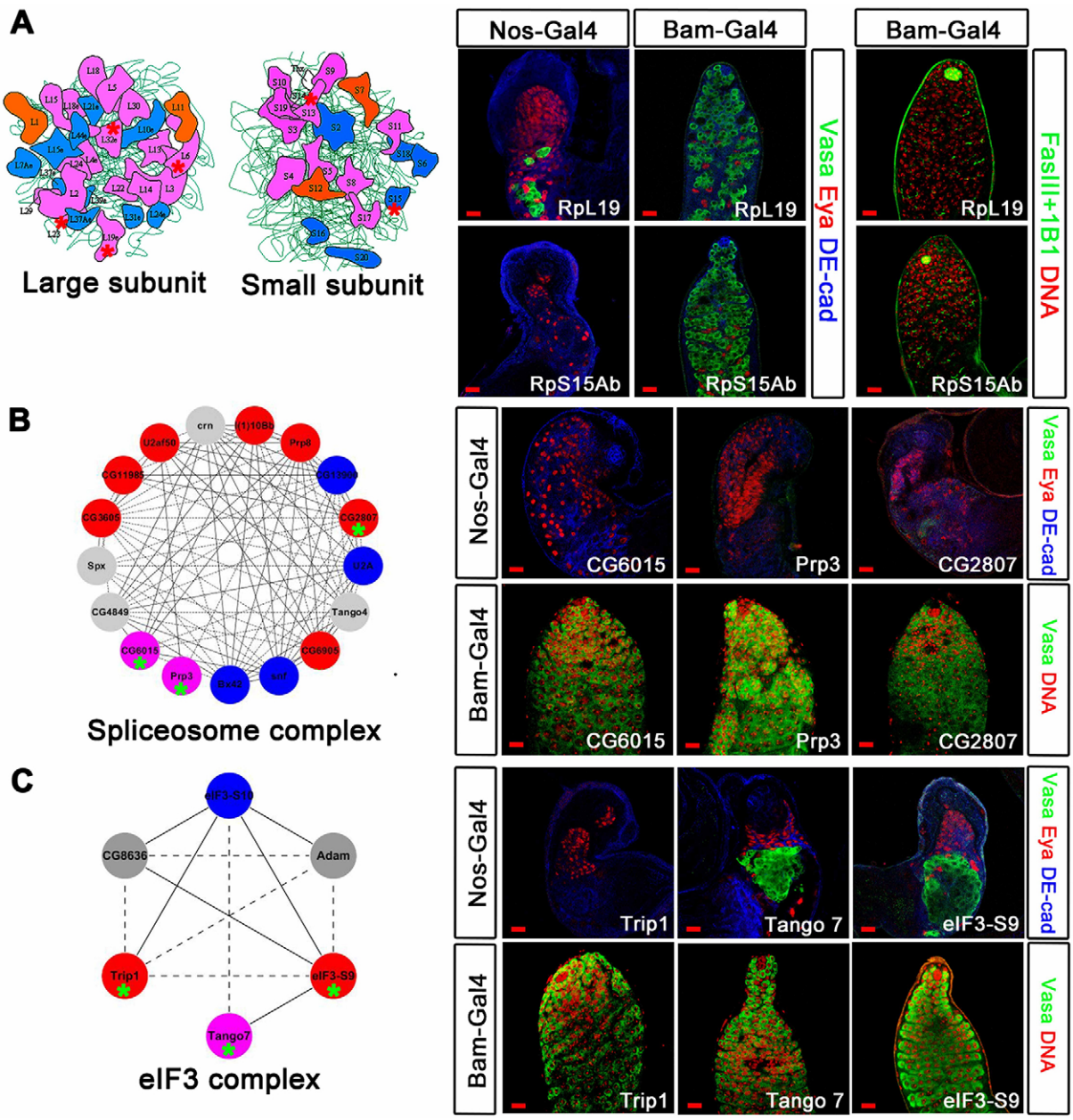

Fig. 3. Regulatory networks of factors involved in GSC self-renewal and differentiation in *Drosophila* testes. (A) Nine genes encoding ribosomal proteins contribute to both GSC self-renewal and TA spermatogonia differentiation. Diagram shows large and small ribosomal subunits (results from the KEGG database). Red stars indicate genes identified in this screen. Knockdown of *RpL19* and *RpS15Ab* with *nos-Gal4* led to depletion of germ cells in testes. RNAi of *RpL19* and *RpS15Ab* with *bam-Gal4* in TA spermatogonia resulted in testes with overproliferation of early germ cells. (B) Four genes (*CG6051*, *prp3*, *CG2807* and *CG6905*) encoding proteins of the spliceosome complex and (C) three genes (*Trip, Tango7* and *eIF3S9*) encode subunits of the eIF3 complex and are required for GSC self-renewal and early germ cell differentiation. The schemes of the complexes were generated by COMPLEAT analysis combined with manual data mining. Red nodes indicate genes identified in the screen. Blue nodes indicate genes that were not identified in this screen. Gray nodes indicate genes that have not been analyzed in this study. All red, blue and gray nodes were generated by automatic COMPLEAT search. The magenta nodes were added manually after a literature survey. The nodes with green stars indicate that the knockdown of the genes causes the phenotypes shown in the right panels. Anti-Vasa, anti-Eya, anti-DE-cad, anti-FasIII and anti-1B1 label germ cells (green in B,C and the left panels of A), differentiated cyst cells (red in the left panels of A and the upper row of B,C), cyst and hub cells (blue in the left part of A and the upper row of B,C), hub cells (green in the right part of A), and fusomes (green in the right part of A), respectively. DNA staining (red in the right part of A and the bottom row of B,C) shows the undifferentiated germ cells. Scale bars: 20 µm.

interactions, two-hybrid interactions, text mining and other data. We further used online software (COMPLEAT) and KEGG pathway analysis to identify the protein complexes. We also performed extensive data mining and manually added proteins missed in the COMPLEAT analysis into complexes.

In total, we identified 99 non-redundant protein complexes required for GSC maintenance in the testis [Table S3 (https://dx.doi.org/10.6084/m9.figshare.3492410.v1)]. The major complexes identified were those participating in processes such as RNA splicing, translation initiation, and proteolysis (Fig. S3). Twenty-nine members of nuclear mRNA splicing complexes and 18 proteins in proteolysis complexes were identified in our screen, suggesting that mRNA splicing and protein degradation are essential for GSC self-renewal.

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Twenty-seven factors were apparently required both for GSC self-renewal and spermatogonia differentiation (i.e. *Hsc70-4*, *Prosbeta-1*, *RpL19*, *RpS15Ab* etc.). To analyze the network of these dual-function genes, we selected these hits and performed complex pathway analysis. Intriguingly, among the 27 hits, 9 were ribosomal proteins (Fig. 3A), 4 participated in mRNA splicing (Fig. 3B) and 3 belonged to the eIF3 complex (Fig. 3C). The enrichment of these genes suggests that mRNA splicing and translation – two key steps of protein synthesis – contribute to both GSC maintenance and early germ cell differentiation.

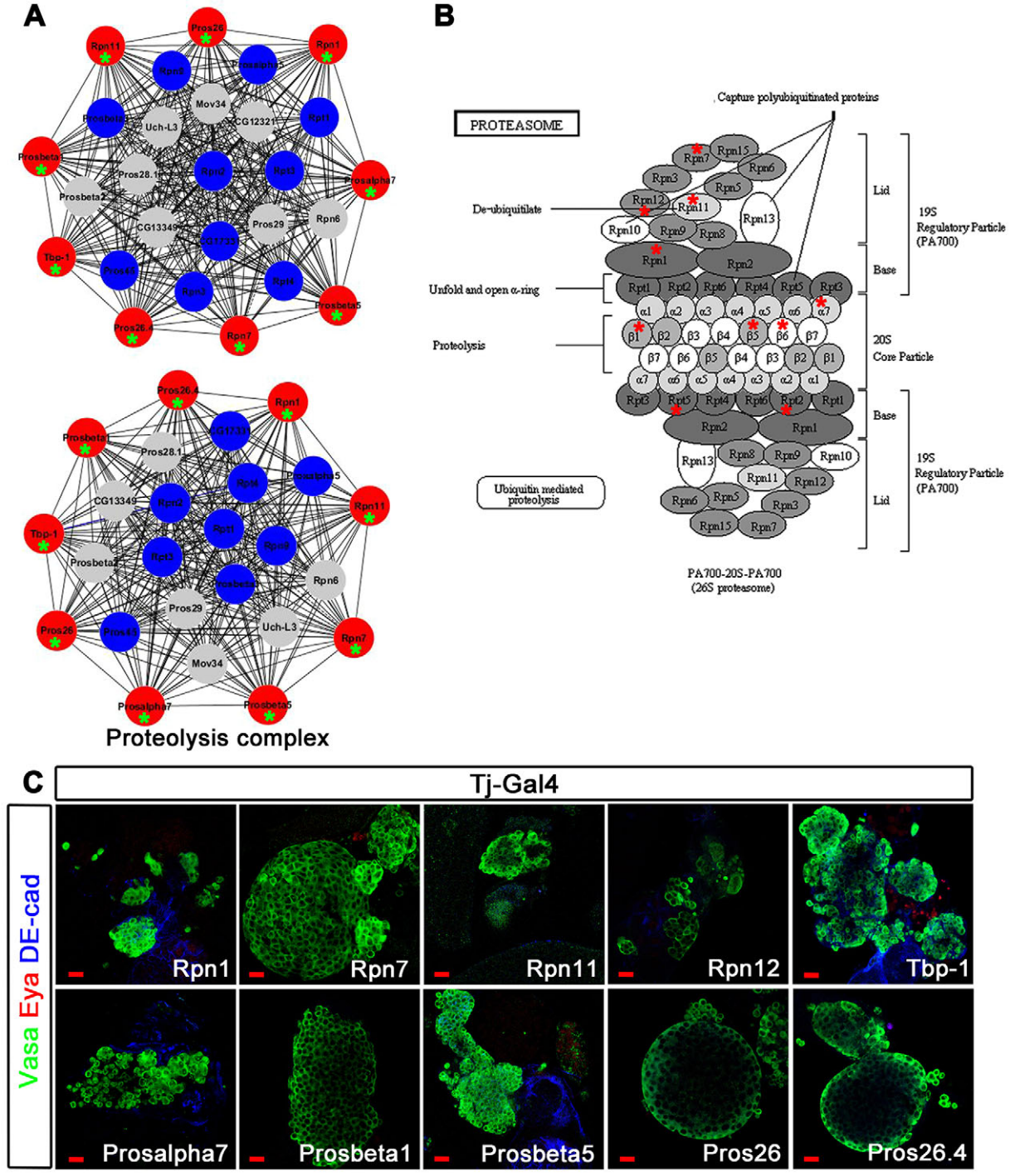

Fig. 4. Knockdown of genes encoding proteasome subunits in cyst cells with *tj-Gal4* leads to the overproliferation of germ cells and testis tumors. (A) Many components of two proteolysis complexes were identified to be required non-cell autonomously for germ cell differentiation. Red nodes indicate genes identified in the screen. Blue nodes indicate genes that were not identified in this screen. Gray nodes indicate genes that have not been analyzed in this study. The nodes with green stars indicate that the knockdown of the genes cause the phenotypes shown in C. (B) A diagram from KEGG analysis indicates the distribution of the subunits identified to be required in cyst cells for germ cell differentiation (red stars). (C) The typical phenotypes of the testis when the indicated genes were knocked down in cyst cells with *tj-Gal4*. Anti-Vasa labels germ cells (green), anti-Eya marks the differentiated cyst cells (red), anti-DE-cad labels the cyst cells and hub cells (blue). Scale bars: 20 µm.

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In the *tj-Gal4* screen, we identified 161 lines that showed loss of most cyst cells and tumor growth in the testes. We also performed complex pathway analysis for these genes, and found that the genes encoding the subunits of a proteasome were enriched in the lines showing tumor growth (Fig. 4A,B). Knockdown of *Rpn1*, *Rpn7*, *Rpn11*, *Rpn12*, *Tbp-1*, *Prosalpha7*, *Prosbeta1*, *Prosbeta5*, *Pros26* and *Pros26.4* in cyst cells led to loss of cyst cells and overproliferation of GSC-like germ cells (Fig. 4C), suggesting that proteasome activity in cyst cells is required for cyst cell survival and promotes germ cell differentiation.

Non-cell autonomous effects of germ cell differentiation

In the screen with *tj-Gal4*, many lines showed testis tumor formation [Table S2 (https://dx.doi.org/10.6084/m9.figshare.3492410.v1)]. We stained these testes with the CySC marker Zfh-1 and the mature cyst cell marker Eya [Table S2 (https://dx.doi.org/10.6084/m9.figshare.3492410.v1), Fig. S5]. About 70.81% (114 of 161) of the testes were negative for both markers (Fig. S5B), indicating the cyst cells are lost. About 20.95% (45 of 161) of the testes were negative for Eya but positive for Zfh1 (Fig. S5C); however, both the number and the distribution of the $Zfh1^{+}$ cells are often abnormal in these testes. This suggested that the proliferation or the differentiation of the CysCs was defective. Among the testes with tumor formation, only one was positive for both Zfh1 and Eya, and the distribution of both markers are largely normal, suggesting this gene might affect the function of CysCs and cyst cells (Fig. S5D). Knockdown of SmB with *tj-Gal4* results in testes that are positive for Eya but negative for Zfh1 (Fig. S5E), indicating this gene might be crucial for CysC maintenance.

Tj-Gal4 is expressed at early developmental stages (Sinden et al., 2012; Leatherman and Dinardo, 2010; Li et al., 2003). Tumor formation could be caused by aberrant germ cell differentiation during development. To test whether RNAi of the same genes at the adult stage could also block GSC differentiation, we knocked down gene expression at the adult stage in cyst cells by using *ptc-Gal4; tubGal80^{ts}*. Eight genes that have the most severe tumor formation phenotypes were selected (Fig. 5A-D, Fig. 4C and Fig. S4D). *Ptc-Gal4; tubGal80^{ts}>UAS-RNAi* flies were cultured at the permissive temperature until eclosure. We then shifted the culture temperature to 28°C and analyzed the testes at 0, 5, 10 and 20 days. Knockdown of two (*dom* and *CG4933*) of the eight genes blocked GSC differentiation and led to testis tumors (Fig. 5F,G). The absence of phenotypes when the other six genes were knocked down with *ptc-Gal4; tubGal80^{ts}* indicates that some genes are required in cyst cells only during the early developmental stages. Alternatively, the difference could be due to the fact that the expression profiles and levels of the *tj-Gal4* and the *ptc-Gal4* drivers are different.

Comparison of regulators for stem cell self-renewal in testis, ovary, intestine and neuroblast

Different stem cells possess some common regulatory mechanisms as well as their own unique features. Thus far, several large-scale RNAi screens have been carried out in various types of stem cells to identify the regulatory networks of self-renewal and differentiation. We therefore compared our screened data with data sets obtained from other stem cell-related screens to identify common or unique factors that regulate self-renewal and differentiation of different stem cells (Neumuller et al., 2011; Yan et al., 2014; Zeng et al., 2015).

We identified 221 hits in our testis screens. The ovary GSC screen identified 365 genes. The ISC and NB screens identified 399 and 620 genes, respectively. A Gene Ontology (GO) term analysis revealed that testis GSCs, ovary GSCs and NBs share most cellular processes in the regulation of self-renewal. In contrast, the cellular processes that regulate self-renewal and differentiation in ISCs are distinctly different (Fig. 6A). Of the 221 genes identified in the testis GSC screen, 95 genes were also identified in the ovarian GSC screen and 47 genes in both the testis GSC and NB screens. Only 27 genes were found in both the testis GSC screen and the ISC screen (Fig. 6B).

To gain further insights into the extent of overlap between the complexes that regulate stem cell maintenance in these systems, we compared the complex analysis data sets of the different screens. GSCs from the ovary and testis commonly required 23 complexes. Testis GSCs and NBs shared 20 complexes. Only one complex was commonly required for both ISCs and testis GSCs (Fig. 6C). Each type of stem cell had many unique regulatory complexes, suggesting that each develops its own combination of regulators.

Five genes, *cdc2*, *tum*, *l(1)10Bb*, *CG6066* and *eIF5*, were identified in all of our stem cell self-renewal screens (Fig. 1F, cdc2; Fig. 2D and Fig. S4A). *cdc2* and *tum* are genes that are required for cell cycle progression and division (Jin et al., 2005; Jones et al., 2010). *l(1)10Bb* and *CG6066* participate in mRNA splicing (Herold et al., 2009). *eIF5* encodes a translation initiation factor (Lasko, 2000). All of these factors participate in basic cellular processes that are commonly required for the self-renewal of stem cells.

In the ovary GSC screen, multiple subunits of the COP9 signalosome (CSN) were identified as key factors that regulate GSC self-renewal (Pan et al., 2014; Yan et al., 2014). We also showed that CSN7 was required for testis GSC self-renewal. In ovaries, CSN7 is only required in the early GSC lineage (Pan et al., 2014). However, knockdown of CSN7 using *bam-Gal4* led to a loss of some germ cells in testes, indicating that CSN7 is not only required in GSCs but also in TA spermatogonia (Fig. S4B).

Many genes involved in ribosome biogenesis were identified in the ovary GSC and NB screens. Knockdown of the expression of most ribosome proteins leads to GSC loss in the ovary and underproliferation of NBs. We also identified many genes that encode ribosome proteins in our testis GSC screen. Knocking down most of these genes with *nos-Gal4* to drive *UAS-shRNA* expression leads to loss of germ cells. Interestingly, we also observed early germ cell differentiation defects when *bam-gal4* was used to knock down gene expression (Fig. 3A, right panels). The detailed mechanisms of the dual functions of these ribosome proteins will require further examination.

Many of the 221 genes identified in our testes GSC screen have never been linked to stem cell self-renewal. For example, we identified several genes encoding metabolic enzymes, including *CG3842*, *CG7910*, *Gdh*, *CG4365*, *CG1236* and *Faa*, which regulated GSC self-renewal (Fig. S4C). We also identified multiple genes encoding mitochondrial proteins that were required for GSC maintenance. The protein encoded by *CG7506* regulates assembly of the mitochondrial proton-transporting ATP synthase complex (Cízková et al., 2008). The protein encoded by *CG11722* is required for assembling the mitochondrial respiratory chain complex I (Saada et al., 2008). Furthermore, the protein encoded by *ND-39* regulates mitochondrial electron transport (Sardiello et al., 2003). These genes have never been identified in other stem cell-related RNAi screens. Interestingly, the loss of several subunits of mitochondrial ATP synthase leads to ovary differentiation defects (Teixeira et al., 2015). We did not identify any gene encoding mitochondrial ATP synthase subunits in our primary morphology screens.

CRL4 E3 ligase complexes are required for GSC self-renewal

Nedd8 is intrinsically required for GSC self-renewal in the ovary (Pan et al., 2014). In our screen, knockdown of *Nedd8* using *nos-*

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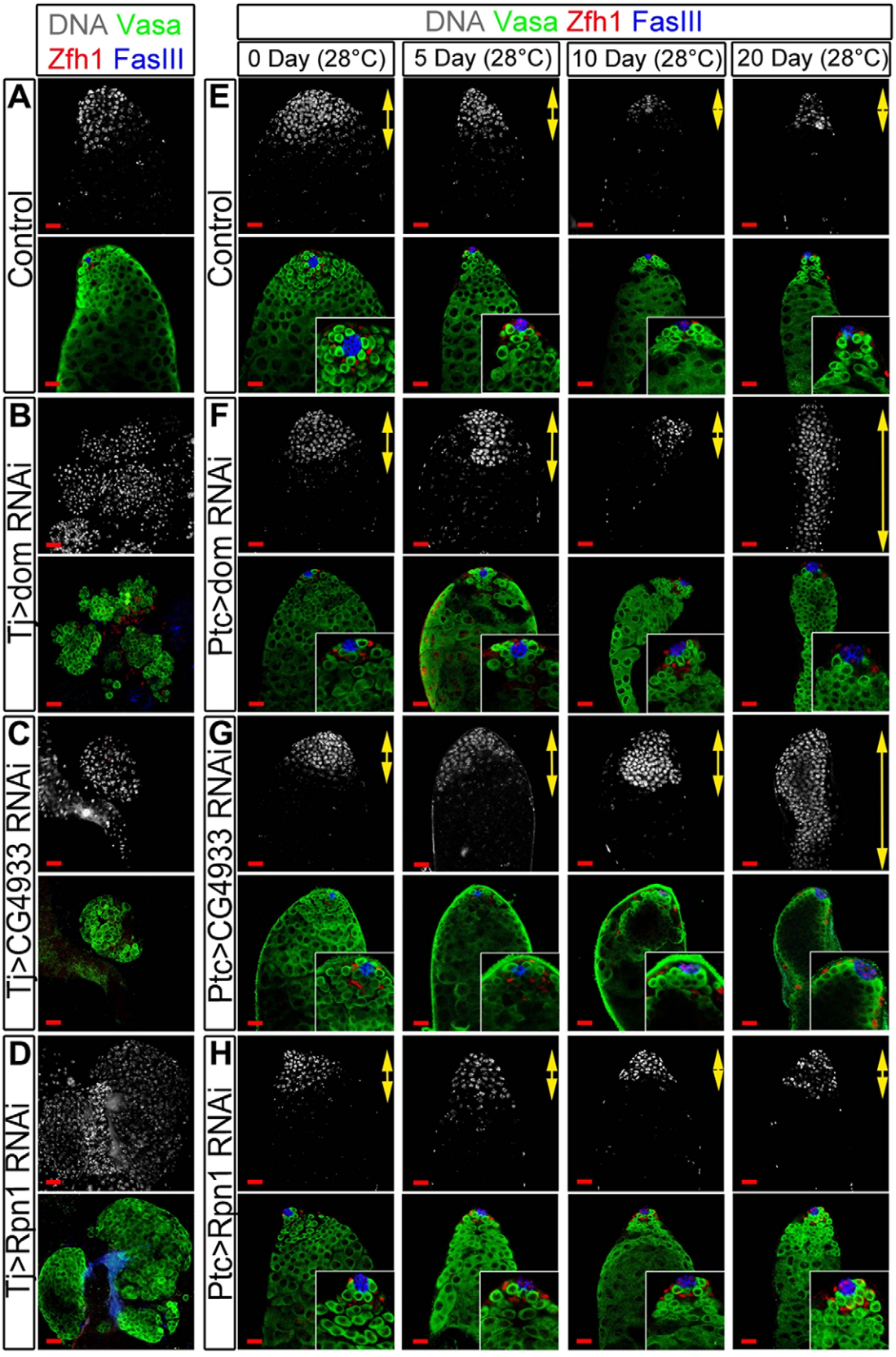

Fig. 5. Non-cell autonomous effects of germ cell differentiation. Knockdown of *dom* (B), *CG4933* (C), and *Rpn1* (D) with *tj-Gal4* resulted in loss of CySCs, overproliferation of germ cells and tumor formation compared with control (A). Knockdown of *dom* (F) and *CG4933* (G) with *ptc-Gal4; tub-Gal80^{ts}* led to non-cell autonomous proliferation of undifferentiated germ cells compared with control (E). RNAi of *Rpn1* (H) with *ptc-Gal4; tub-Gal80^{ts}* did not cause a testis defect. Anti-Vasa (green), anti-Zfh-1 (red), anti-FasIII (blue) and DNA staining (gray) labeled germ cells, CySCs, hub cells and undifferentiated germ cells, respectively. Yellow arrows represent regions of undifferentiated germ cells. Scale bars: 20 µm.

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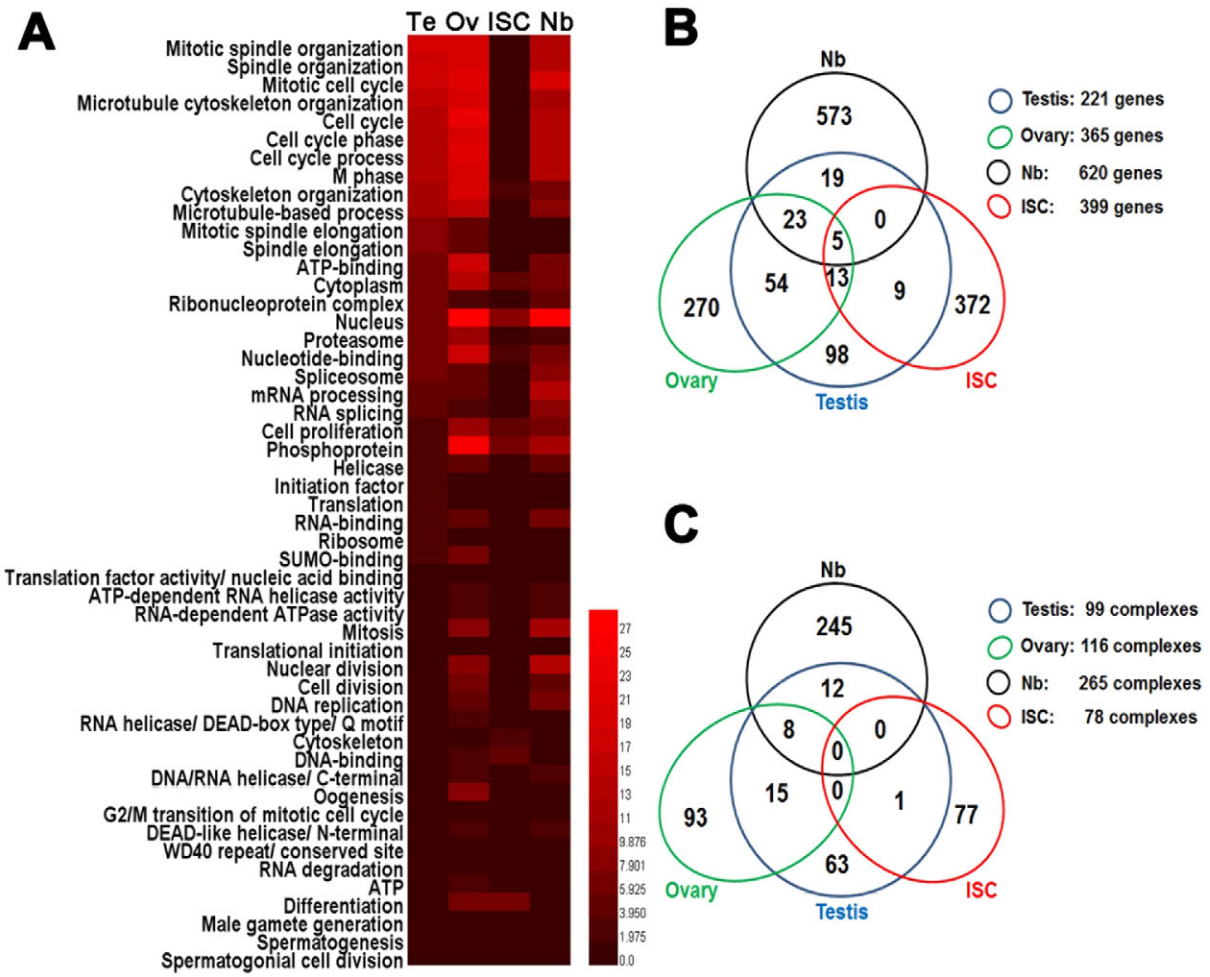

Fig. 6. Comparison of regulatory networks for stem cell self-renewal in testis, ovary, intestine and neuroblast. (A) Heat map displaying over- and under-representation of selected GO terms in genes identified in the testis, ovary, ISC and NB RNAi screens. (B) Numbers of the genes identified in testis, ovary, ISC and NB RNAi screens. (C) Numbers of complexes identified in testis, ovary, ISC and NB RNAi screens.

Gal4 resulted in testes without germ cells (Fig. 7B), suggesting that *Nedd8* is a key factor required for testis GSC maintenance. *Nedd8* is an ubiquitin-like protein that activates the ubiquitin E3 ligase family, the cullin-RING ligases (CRLs) (Lammer et al., 1998). CRLs are protein complexes with an elongated horse shoe-like structure (Zimmerman et al., 2010). One of the cullin proteins (5 in flies and 7 in mammals) forms a central CRL scaffold that links to E2 through a ring-finger protein and binds to the substrate by receptor modules. How *Nedd8* regulates GSC self-renewal is unknown. Interestingly, only the knockdown of *Cullin 4* (*Cul-4*), but not other cullin proteins, using *nos-Gal4* resulted in tiny testes without germ cells (Fig. 7C), suggesting that the Cullin 4-RING ligase (CRL4) complex (Fig. 7A) might be the only CRL that mediates the functions of *Nedd8* in testis. The substrate recognition module of CRL4 comprises a linker protein Damage-specific DNA binding protein 1 (DDB1) and the WD40 domain-containing DCAF proteins as substrate receptors. *Piccolo* (*pic*), the *Drosophila Ddb1* ortholog, was also identified in our screen. RNAi of *pic* with *nos-Gal4* (Fig. 7D) led to testis phenotypes identical to those of *Nedd8* and *Cul-4* knockdown animals. In addition, 13 WD40 domain-containing proteins were also required for GSC self-renewal in testes (Table S4). The knockdown of *mahjong* (*mahj*) (Fig. 7E), *WD repeat domain 82* (*Wdr82*) (Fig. 7F) and *Chromatin assembly factor 1* (*Caf1*) (Fig. 7G) with *nos-Gal4* led to a complete depletion of germ cells. RNAi of *will die slowly* (*wds*) with *nos-Gal4* resulted in the loss of most germ cells (Fig. 7H). Proteins encoded by the mammalian othologs of *mahj*, *Wdr82*, *Caf1* and *wds* were reported to function as DDB1-CUL4-associated factors (DCAFs) that recognize substrates for CRL4-mediated ubiquitylation (Angers et al., 2006; Lee and Zhou, 2007).

When the CRL4 components we identified are knocked down in TA spermatogonia with *bam-Gal4* or in the cyst cells with *tj-Gal4*, the phenotypes of animals differ depending on the component knocked down. This is not surprising, considering the diversity of substrates of the CRL4 complex and different roles of different CRL4 components. Knockdown of *pic* (Fig. 7D′), *wds* (Fig. 7H′), and *Caf1* (Fig. 7G′) with *bam-Gal4* resulted in no obvious testis defects, whereas RNAi of *pic* (Fig. 7D″), *wds* (Fig. 7H″), and *Caf1* (Fig. 7G″) with *tj-Gal4* led to abnormal testes. These results suggest that the genes are not merely essential for cell survival. However, knockdown of *Nedd8* (Fig. 7B-B″), *Cul-4* (Fig. 7C-C″), *mahj* (Fig. 7E-E″) and *Wdr82* (Fig. 7F-F″) with *nos-Gal4*, *bam-Gal4* and *tj-Gal4* all resulted in defective testes, suggesting that these genes play multiple roles in different cells.

To examine whether CRL4 components are also required for GSC maintenance in adult testes, we also generated *Cul-4*, *pic* and *mahj* mutant clones in adult testes and examined the self-renewal of GSCs. *Cul-4*^{KG02900}, two *pic* mutant alleles (*pic*^{GE28589} and *pic*^{EY01408}) and

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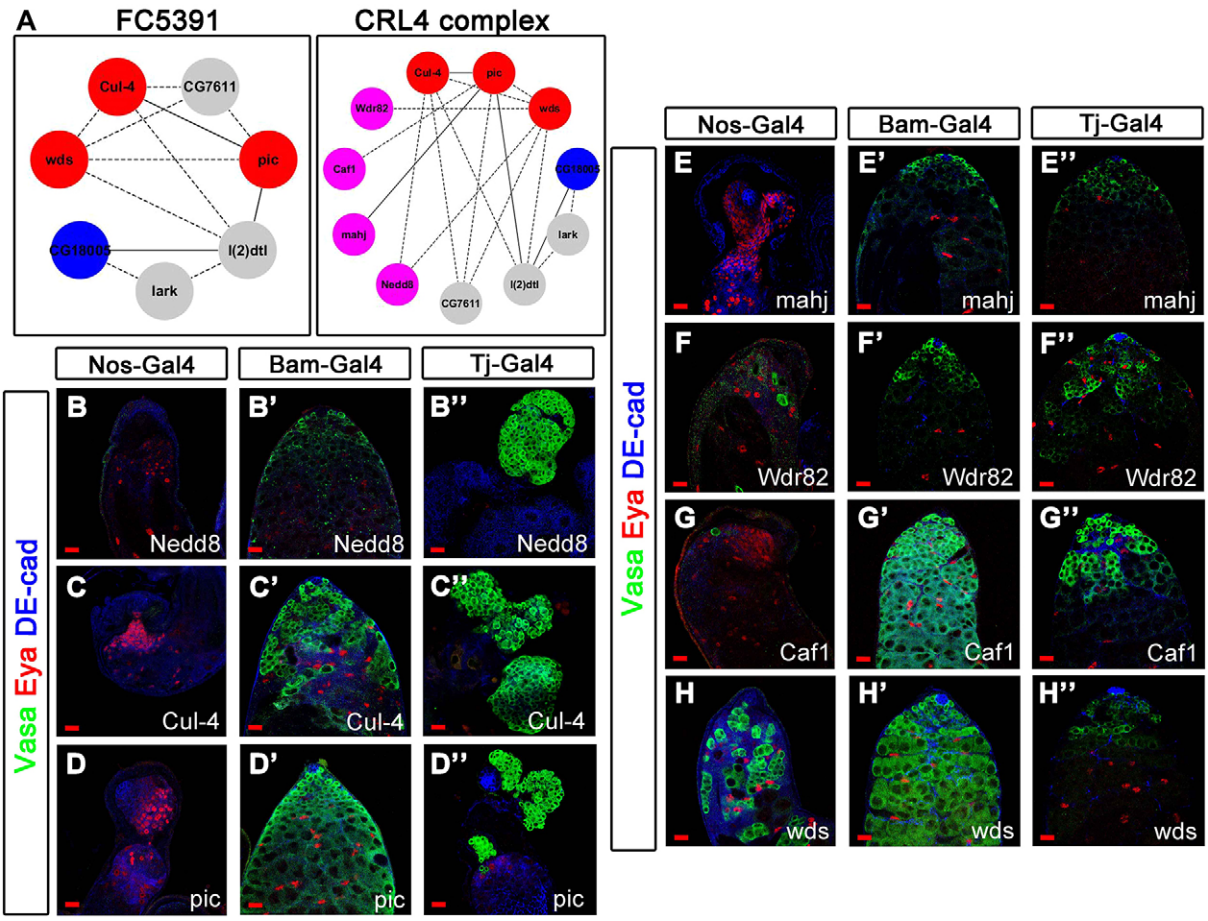

Fig. 7. CRL4 complexes are required for GSC self-renewal in *Drosophila* testis. (A) Two complexes containing CRL4 components were identified by COMPLEAT analysis and manual data mining. Red and magenta nodes were identified in this screen whereas blue nodes were not. Gray nodes indicate genes that have not been analyzed in this study. Red, blue and gray nodes were generated by automatic COMPLEAT analysis; magenta nodes were added manually on the basis of the literature. (B-H″) Genes (*Nedd8*, *Cul-4*, *pic*, *mahj*, *Wdr82*, *Caf1* and *wds*) encoding components of the CRL4 complex were required for GSC self-renewal. Anti-Vasa, anti-Eya and anti-DE-cad label germ cells (green), differentiated cyst cells (red) and cyst cells or hub cells (blue), respectively. Scale bars: 20 µm.

mahj^{LL00674} were used to generate mutant clones. Subsequently, the numbers of GFP-negative GSC and SP clones were examined at different time points. For *Cul-4* mutants, the GSC clones disappeared within 2 days of clone induction (Fig. 8C,D). The GSC clones of *pic* and *mahj* mutants were completely lost at day 15 after clone induction, whereas more than half of the WT control clones still existed (Fig. 8A,B,E,F). These data suggest that the CRL4 complex is also required for adult GSC self-renewal and maintenance.

DDB1 is required for the maintenance of SSCs in mice

The CRL4 E3 ligases in mammals are more complicated than those in flies. There are two CUL4 proteins, CUL4A and CUL4B, which play redundant roles as scaffold proteins in substrate ubiquitylation. Although *Cul4a*-knockout male mice are infertile and exhibit severe deficiencies in germ cell meiosis, they do not show defects of SSC maintenance (Kopanja et al., 2011; Yin et al., 2011). Whether or not CRL4 is required for SSC maintenance remains unknown, but there could be redundancy of *Cul4a* and *Cul4b*. The mammalian genome includes only one *Ddb1* gene encoding a core component of the CRL4 complex. Therefore, to analyze whether the function of the CRL4 complex in GSCs is evolutionarily conserved, we chose to analyze the function of *Ddb1* in mouse SSCs.

We generated germ cell-specific *Ddb1*-knockout mice by crossing *Ddb1*$^{fl/fl}$ mice with *Ddx4-Cre* transgenic mice (Yu et al., 2013). Testes were much smaller in 2-month-old *Ddb1*$^{fl/-}$*; Ddx4-Cre* mice compared with controls (Fig. 9A). Immunofluorescence (IF) analysis of the testes showed an absence of the germ cell marker MVH (Fig. 9B). Hematoxylin and eosin (H&E) staining indicated that germ cells were depleted in *Ddb1*$^{fl/-}$*; Ddx4-Cre* testes and epididymides (Fig. 9C,D). Consistent with these data, male *Ddb1* conditional knockout (cKO) mice were sterile, as indicated by the failure of egg fertilization (Fig. 9E) after successful mating (Fig. 9F).

To determine whether the loss of germ cells in *Ddb1* cKO mice is due to SSC maintenance defects, we analyzed the testes of newborn mice at different time points. We stained testis sections with a germ cell marker MVH, a SSC marker PLZF and a Sertoli cell marker WT1 at postnatal day (PD) 3, 5, 7, 9 and 15 (Fig. 10A-D). At PD3, the number of PLZF-labeled SSCs was largely identical between the control and *Ddb1* cKO mice. At PD5, the number of SSCs in the testes of control but not *Ddb1 c*KO increased. At PD7 and PD9, the number of SSCs in the control testes further increased, although the number of SSCs in the *Ddb1* cKO mice declined. At PD15, the SSCs had completely disappeared in *Ddb1* cKO mice (Fig. 10B,D). MVH-labeled germ cells in the control testes reliably increased during early postnatal development. In contrast, the number of germ cells decreased in the *Ddb1* cKO mice. At PD9, no MVH-positive cells were detected in the *Ddb1* cKO testes (Fig. 10A). In contrast to

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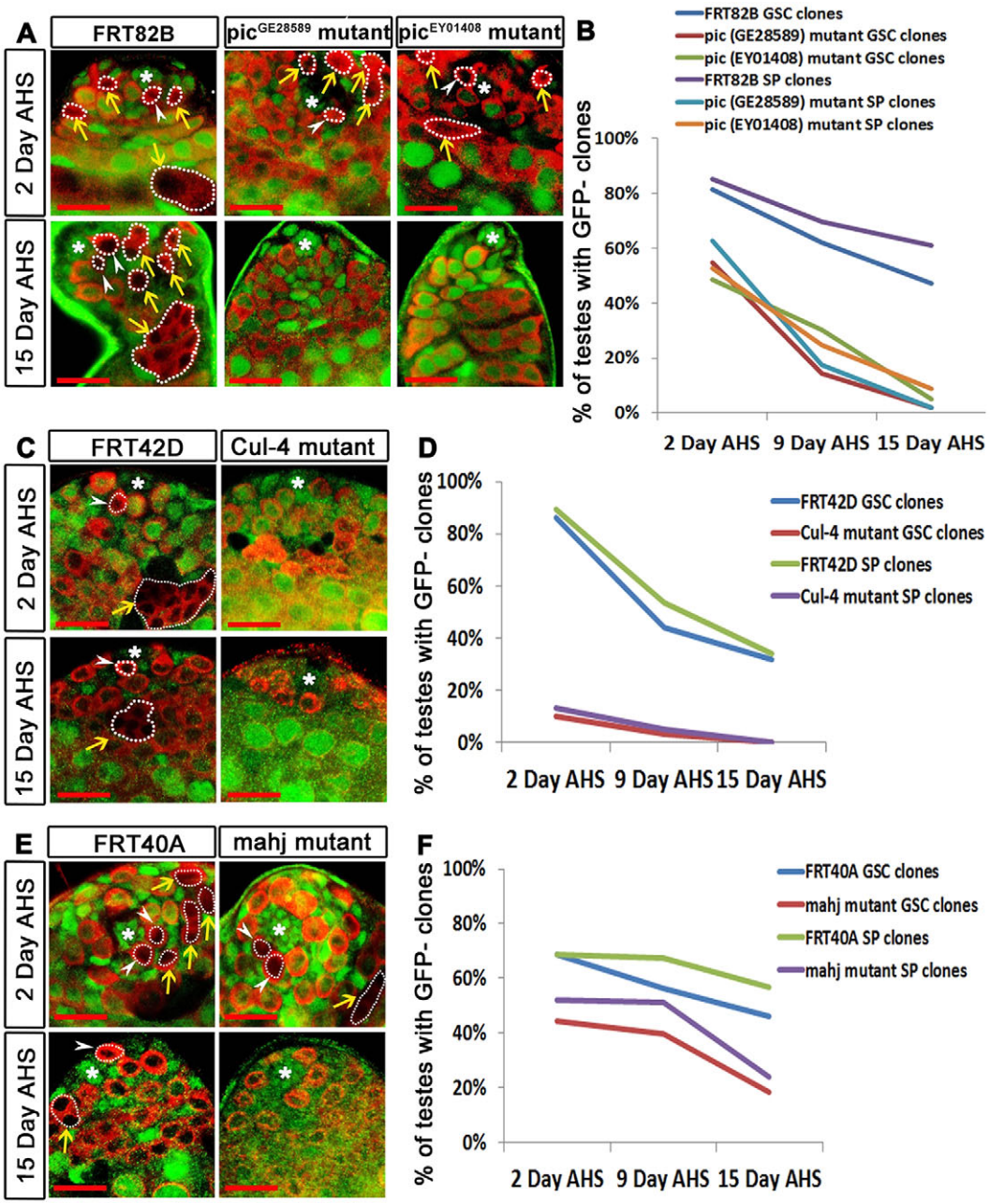

Fig. 8. CRL4 complexes are required for GSC maintenance in the adult *Drosophila* testis. (A-F) Mosaic analysis of *pic* (A,B), *Cul-4* (C,D) and *mahj* (E,F) mutants shows that core components of CRL4 complex are required for GSC maintenance in adult testes. Mutant clones were outlined and identified by the absence of GFP. White arrowheads indicate GSC clones and yellow arrows indicate spermatogonia (SP) clones. (B,D,F) The percentage of testes with control and mutant GSC and SP clones maintained post clonal induction. AHS, after heat shock. Anti-GFP labeling shows the ubi-GFP expression pattern (green). Anti-Vasa labels germ cells (red). The hub cells are marked with white stars. Scale bars: 20 µm.

the decline of the SSCs and germ cells, there was no difference in the numbers of Sertoli cells in the WT and *Ddb1* cKO testes (Fig. 10C). These results suggest that *Ddb1* is required for SSC maintenance in the mouse testis.

DISCUSSION

The *Drosophila* testis provides an excellent model to study stem cell niches (Hu et al., 2014; Yu et al., 2015). Until now, no systematical analysis of the regulatory network of GSCs in the testis has been conducted. Here, we identified 221 genes that regulate GSC self-renewal or differentiation, many of which were identified for the first time. The factors that regulate protein synthesis and degradation are enriched in these 221 hits, suggesting that protein homeostasis is essential for GSC self-renewal and GSC progeny differentiation.

The COP9 signalosome governs GSC self-renewal and differentiation in the ovary. The COP9 complex, which is composed of eight CSN subunits (CSN1-CSN8), removes Nedd8 modifications from its target proteins (Pan et al., 2014). Activity of the COP9 complex is essential for GSC self-renewal. Interestingly, CSN subunits, with the exception of CSN4, are also required for GSC progeny differentiation (Pan et al., 2014). In this study, we also identified CSN7 and Nedd8 as GSC self-renewal factors. However, we did not observe the differentiation function of these two genes in

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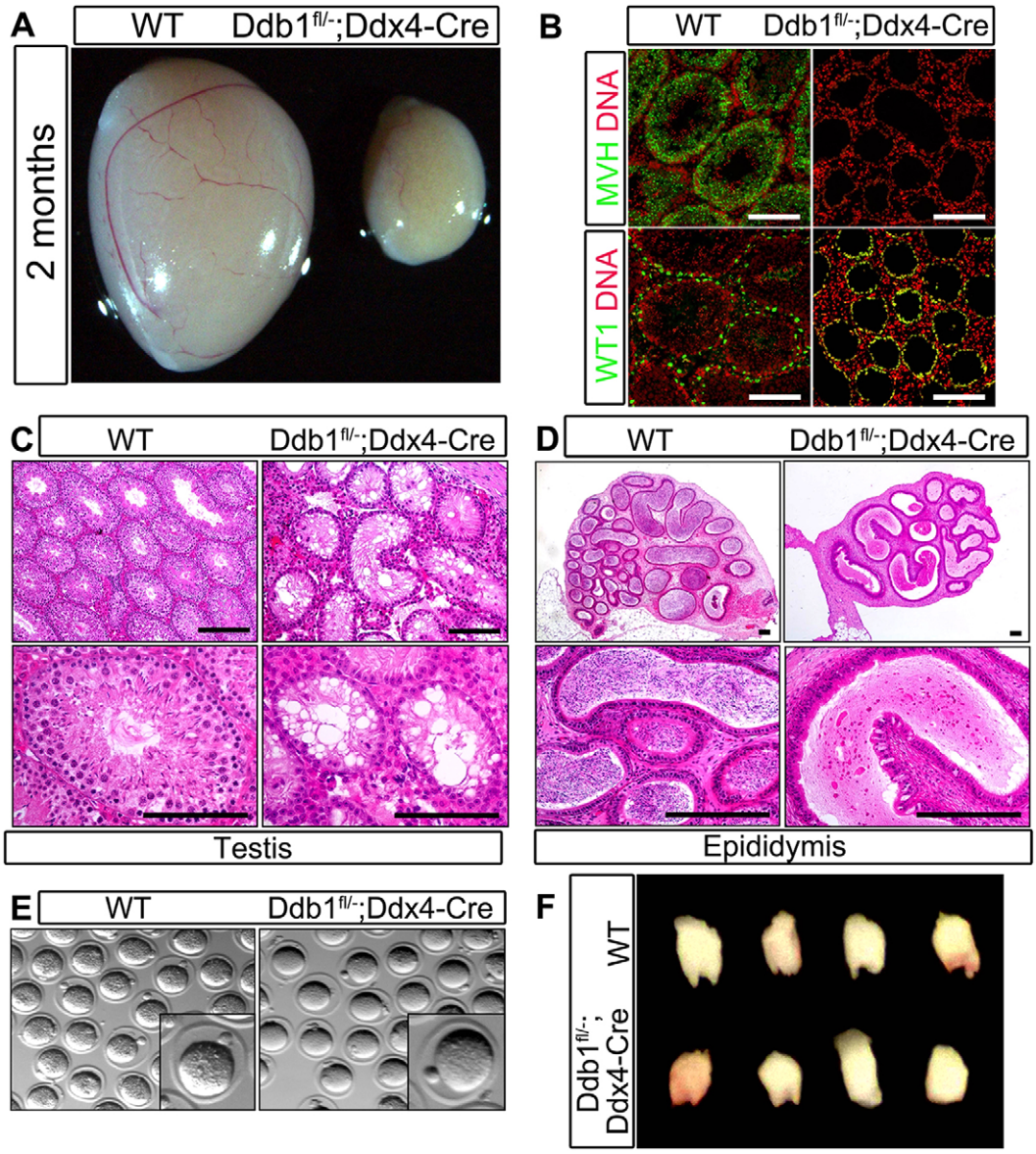

Fig. 9. *Ddb1* is required for spermatogenesis in mice. (A) Germ cell-specific knockout of *Ddb1* leads to small testes in mice. (B) Germ cells labeled with MVH (green in top row) disappeared, whereas Sertoli cells labeled with WT1 (green in bottom row) were intact in seminiferous tubules of *Ddb1*$^{fl/-}$*; Ddx4-Cre* mice. DNA was stained with DAPI (red). (C,D) HE staining of testis (C) and epididymis (D) showed that germ cells completely disappeared in *Ddb1*$^{fl/-}$*; Ddx4-Cre* mice testes. (E) WT females could not produce fertilized eggs (indicated by pronucleus formation) after mating with *Ddb1*$^{fl/-}$*; Ddx4-Cre* males. (F) Vaginal plugs form in WT females after mating with WT or *Ddb1*$^{fl/-}$*; Ddx4-Cre* males, indicating that both males can successfully mate with females. Scale bars: 100 µm.

the testis. In our case, RNAi of *CSN7* and *Nedd8* with *nos-Gal4* resulted in testes with no germ cell (Fig. 7B and Fig. S4B). Knockdown of *CSN7* and *Nedd8* with *bam-Gal4* in TA spermatogonia also led to a loss of germ cell cysts (Fig. 7B′ and Fig. S4B), suggesting that the COP9 complex might regulate GSCs in testes differently than in ovaries.

The major substrates of COP9 complexes are Cullin-RING E3 ligase complexes. There are five Cullin proteins in flies. Interestingly, we found that only Cul-4 is required for GSC self-renewal. CRL4 complexes may mediate the functions of COP9 in GSCs. In this study, we not only identified the scaffold and linker proteins Cul4 and Pic, respectively, but also found multiple WD40 domain-containing proteins as potential substrate recognition adaptors. Furthermore, *Ddb1*, the mammalian ortholog of *pic*, also plays an important role in maintaining SSCs in mouse testes. CRL4 has been shown to regulate epigenetic factors in mouse oocytes and early embryos (Yu et al., 2013). The functions of CRL4 in mouse oocytes are mediated by the CRL4 substrate adaptor DCAF1 (also known as VPRBP). Our screening results indicated that *mahj*, the *Drosophila Dcaf1* homolog, was also essential for GSC maintenance. Collectively, these studies indicate that CRL4 and DCAF1 possess an evolutionarily conserved role of considerable importance in germ cell development.

In this study, we showed that genes encoding nine ribosome proteins, four mRNA splicing proteins and three eIF3 complex proteins were not only required for GSC self-renewal but are also essential for spermatogonial differentiation. Translational regulation is crucial for GSC self-renewal in both testes and ovaries (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Shen et al., 2009; Spradling et al., 2011; Wang and Lin, 2004). In fly ovaries, it has

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been well established that translational repression plays a major role in making the decision between self-renewal and differentiation (Slaidina and Lehmann, 2014). However, most factors identified so far either play a role in GSC self-renewal or GSC progeny differentiation. The dual functions of the genes identified here suggest that translation regulation is complex and crucial in germ cells. During the revision of this manuscript, Sanchez and colleagues published a paper showing that ribosome biogenesis and protein synthesis are required for GSC transition from self-renewal to differentiation in fly ovary (Sanchez et al., 2016).

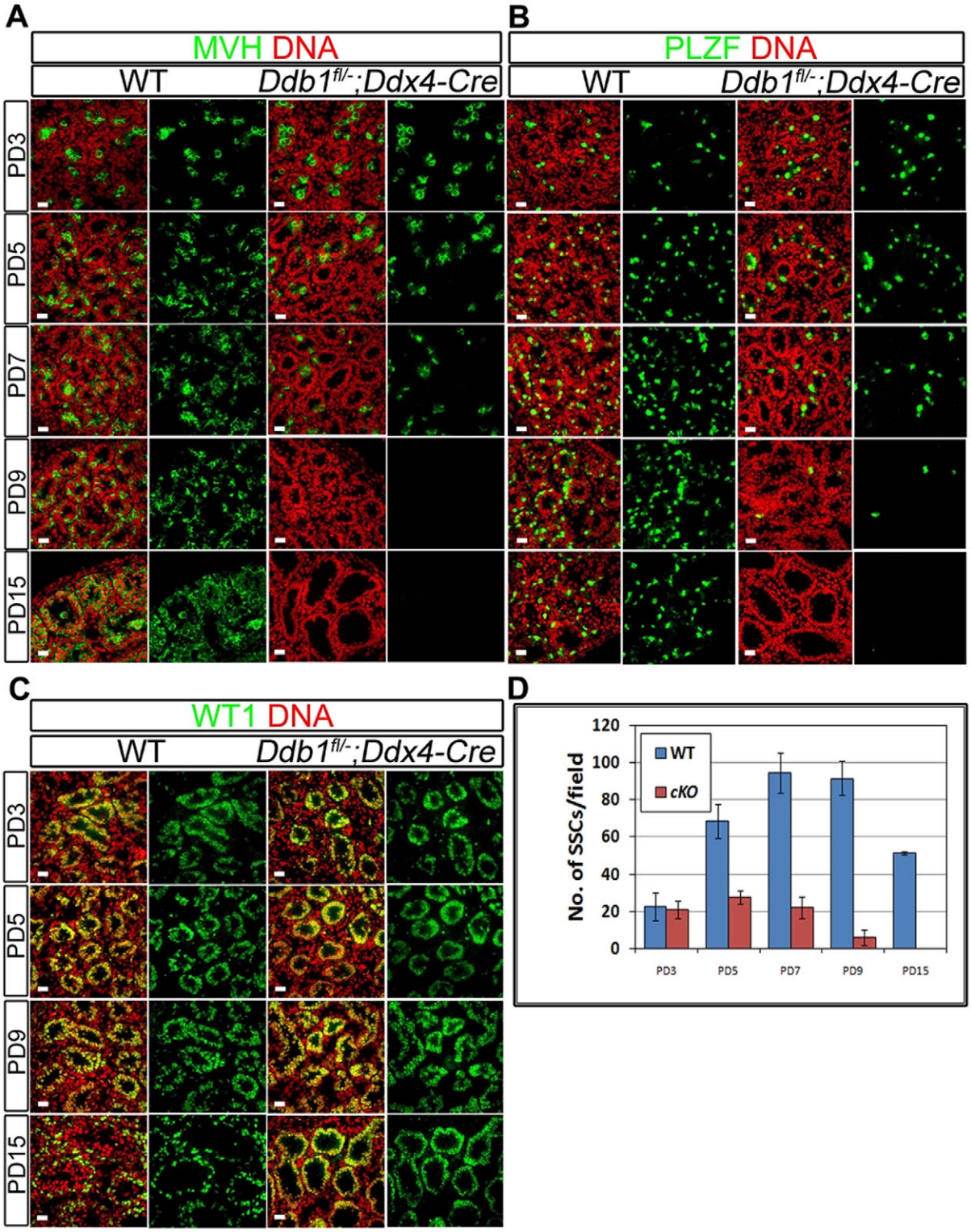

Fig. 10. *Ddb1* is essential for SSC maintenance in the mouse testis. Germ cells (A) and SSCs (B) but not Sertoli cells (C) are gradually lost in the *Ddb1*$^{fl/-}$*; Ddx4-Cre* mice testes. (D) Number of SSCs in WT and *Ddb1*$^{fl/-}$*; Ddx4-Cre* testes. MVH (green in A) marks germ cells, PLZF (green in B) labels SSCs, WT1 (green in C) marks Sertoli cells. DNA is stained with DAPI (red in A-C). PD: postnatal day. Scale bars: 20 µm.

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Together with our data, this suggests that translation regulation is critical for GSC differentiation in both males and females.

The identification of the eIF3 complex is particularly interesting. During translation initiation, this complex mediates recruitment of the 40S ribosomal subunit to the 5′UTRs of mRNAs (Walsh and Mohr, 2014). A recent study showed that eIF3 was more than a translation initiation scaffold; it also directed the translation of specific cellular mRNAs by binding a structured RNA element (Lee et al., 2015). The eIF3 complex has been linked to various cancers (Hershey, 2010). Thus, the self-renewal and differentiation functions of this complex might be a common theme in stem cells.

The switch from TA proliferation to differentiation in the *Drosophila* testis is mediated by translational control of Mei-P26 and Bam (Chen et al., 2014; Insco et al., 2012; Li et al., 2012). Mei-P26 could be a key molecule regulated by the translational machinery we identified here.

When gene expression in cyst cells is knocked down by *tj-Gal4*, many lines show a loss of cyst cells and overproliferation of GSC-like cells, which lead to tumor formation. Cyst cells are reportedly required for germ cell differentiation and a loss of cyst cells in the adult stage leads to overproliferation of early germ cells (Lim and Fuller, 2012). Tumor formation in *tj-Gal4>UAS-RNAi* lines may be due to the loss of cyst cells. The overproliferation of GSCs suggests that cyst cells either provide the initiation signals for germ cell differentiation or constrain the self-renewal signals from the hub cells. Surprisingly, *tj-Gal4>UAS-RNAi* testes with GSC overproliferation are devoid of distinguishable hub cells in many cases, suggesting that the overproliferated GSC-like cells are self-sustained.

In summary, our systematic analysis of genes that regulate GSC self-renewal and differentiation in *Drosophila* testes provides a rich resource to understand spermatogenesis in mammals and stem cell biology in general.

MATERIALS AND METHODS

Mouse strains and crosses

Wild-type *C57/B6* mice were obtained from the Zhejiang Academy of Medical Science, China. *Ddb1*$^{flox/flox}$ and *Ddx4-Cre* mice were previously generated (Cang et al., 2006; Gallardo et al., 2007). Mice were maintained under SPF conditions in a controlled environment of 20-22°C, with a 12 h:12 h light:dark cycle, 50-70% humidity, and food and water was provided ad libitum. Animal care and experimental procedures were in accordance with the Animal Research Committee guidelines of Zhejiang University.

Mice lacking *Ddb1* in their germ cells were generated by crossing *Ddx4-Cre* mice with *Ddb1*$^{flox/flox}$ mice. All mutant mouse strains had a *C57/B6* background. To test fertility, control and *Ddb1* cKO male mice were mated with 10- to 12-week-old fertile WT females overnight. Successful mating was confirmed by the presence of vaginal plugs. Zygotes were harvested from oviducts at 20 h after hCG injection and pronucleus formation was examined in the zygotes.

Mosaic clonal analysis

GSC and SP clones were induced with the FLP/FRT-mediated mitotic recombination technique (Xu and Rubin, 1993) in flies with genotypes as listed in supplementary Materials and Methods.

RNAi screen strategies and fly strains

The transgenic RNAi flies used in the screen were ordered from the Tsinghua Fly Center (THFC) and were from the same RNAi collection as the TRiP (Transgenic RNAi Project). The crosses were set and raised at room temperature (25°C). Male *Gal4* drivers were crossed to the transgenic *UAS-RNAi* virgin females. For germ cell RNAi, one copy of *UAS-Dcr-2* was used together with the *Gal4* lines. The male progenies were dissected for light microscopy. The animals with small testes were picked and further processed for confocal analysis.

Temperature-sensitive *ptc-Gal4* flies were crossed with *UAS-RNAi* lines at 25°C. After egg laying, cultures were transferred to 18°C until adults emerged. Newly enclosed males were shifted to high temperature (29°C) for 0, 5, 10 and 20 days followed by dissection, staining and confocal analysis.

Large-scale RNAi screen and fly strains

All *UAS-RNAi* transgenic fly strains are available from the THFC (Ni et al., 2011). For the genome-wide RNAi screen, we used *Gal4* expressed in the testis to drive the expression of *UAS-RNAi* in different cell types (Ni et al., 2008). The following *Gal4* lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC) and the *Drosophila* Genetic Resource Center (DGRC): *nos-Gal4* (BDSC, #4937), *tj-Gal4* (DGRC, #104055), *UAS-Dcr-2* (BDSC, #24650, #24651). *Bam-Gal4* was a gift from D. H. Chen (Institute of Zoology, Chinese Academy of Sciences). The membrane-localized GFP line was obtained from BDSC: *UAS-mCD8-GFP* (BDSC, #5130). The temperature-sensitive *ptc-Gal4* was from THFC: *w; ptc-Gal4; Gal80ts/TM6b* (THFC, #THJ0203). The following mutant alleles were from DGRC and BDSC: *Cul-4*^{KG02900} (DGRC, #111538), *pic*^{GE28589} (BDSC, #26890), *pic*^{EY01408} (BDSC, #15350) and *mahj*^{LL00674} (DGRC, #140129).

Immunocytochemistry and microscopy

Immunocytochemistry and visualization of fly and mouse testes was carried out using standard techniques with antibodies as described in the supplementary Materials and Methods.

Bioinformatics analysis

The size (small or tiny) of the testis was judged by the length of the testis, which was measured using ImageJ (imagej.nih.gov/ij). Complex analysis was done using COMPLEAT (http://www.flyrnai.org/compleat) according to previous studies (Vinayagam et al., 2013; Yan et al., 2014). The non-redundant protein complexes that were over-represented among the genes were scored by comparing with the experimental background, with a *P*-value cut-off of 0.05. An interaction matrix was established amongst all genes that scored in the screen and the resulting network was visualized using Cytoscape. Databases of protein and genetic interactions used in this article were DroID (http://www.droidb.org) and InterologFinder (http://www.interologfinder.org). Diagrams indicating the distribution of the subunits of ribosome and proteasome are adopted from the KEGG database (http://www.kegg.jp).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed the experiments: C.T., H.-Y.F., J.Y. Performed the experiments: J.Y., X.L., X.C., C.Y., Y.X., Y.L., L.X. Analyzed the data: C.T., H.-Y.F., J.Y., C.Y. Wrote the paper: C.T., H.-Y.F., J.Y.

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Data availability

Supplementary Tables S1-S3 are available via Figshare at https://dx.doi.org/10.6084/m9.figshare.3492410.v1

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.134247.supplemental

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Supplemental Materials and Methods

Mosaic clonal analysis

GSC and SP clones were induced with the FLP/FRT-mediated mitotic recombination technique (Xu and Rubin, 1993) in files with following genotypes:

yw, hs-FLP/Y; ubiGFP, FRT82B/FRT82Biso (CTL);

yw, hs-FLP/Y; ubiGFP, FRT82B/FRT82B $pic^{GE28589}$;

yw, hs-FLP/Y; ubiGFP, FRT82B/FRT82B $pic^{EY01408}$;

yw, hs-FLP/Y; ubiGFP, FRT40A/FRT40Aiso(CTL);

yw, hs-FLP/Y; ubiGFP, FRT40A/FRT40A $mahj^{LL00674}$;

yw, hs-FLP/Y; ubiGFP, FRT42D/FRT42Diso(CTL);

yw, hs-FLP/Y; ubiGFP, FRT42D/FRT42D $Cul\text{-}4^{KG02900}$;

To induce clones, 0– to 2–day-old adult male flies were subjected to 1 hr heat shock at 37℃. Flies were kept at 25℃ and dissected and stained at 2–, 9– and 15–days after heat shock (AHS). GSC clones were identified as Vasa-positive, GFP-negative cells attaching to the hub cells.

Mouse strains and crosses

Wild type *C57/B6* mice were obtained from the Zhejiang Academy of Medical Science, China. *Ddb1*$^{flox/flox}$ and *Ddx4-Cre* mice were previously generated (Cang et al., 2006; Gallardo et al., 2007). Mice were maintained under SPF conditions in a controlled environment of 20–22℃, with a 12/12 h light/dark cycle, 50–70% humidity, and food and water provided ad libitum. Animal care and experimental procedures were in accordance with the Animal Research Committee guidelines of Zhejiang University. Mice that lacked *Ddb1* in their germ cells were generated by crossing *Ddx4-Cre* mice with previously reported *Ddb1*$^{flox/flox}$ mice. All mutant mouse strains were had a*C57/B6* background.

Mice fertility test

To test fertility, control and *Ddb1 cKO* male mice were mated with 10– to 12–week old fertile wild type females overnight. Successful mating was confirmed by the presence

of vaginal plugs. Zygotes were harvested from oviducts at 20 hours after hCG injection. And pronucleus formation was examined in the zygotes.

Hematoxylin and eosin (H&E) staining

Testes tissues were fixed overnight in 10% PBS buffered formalin, dehydrated using an ascending series of graded ethanol solutions, and then embedded in paraffin. Testes samples were serially sectioned at 5 μm thickness and stained with haematoxylin and eosin.

Light and phase-contrast microscopy

Fly testes were dissected in 1x phosphate-buffered saline (PBS) and washed several times. For an overall view, the testis was observed directly under light microscope. Shredded testes were observed on slides by a phase-contrast microscope after gently squashing them with a cover slip. All stages of germ cells during spermatogenesis are found in normal testes.

Immunofluorescence and antibodies

Fly testes were dissected in 1x PBS and fixed for 30 min in 4% paraformaldehyde. After washing three times in 1x PBS with 0.1% Triton X–100 (PBST) and blocking for 1hr in 5% bovine serum albumin (BSA), the samples were incubated with primary antibodies overnight at 4℃. After washing three times for 10 min in 0.1% PBST, the samples were incubated for 1 hr with secondary antibodies at room temperature followed by three times washing in 0.1% PBST. Testes were then stained with Hoechst 33342 (1.0 mg/ml, Invitrogen) for 5 min before mounting.

For mice testis staining, the tissues were fixed in 4% paraformaldehyde, embedded in O.C.T. compound (Sakura Finetek USA Inc.), and stored at –80℃ before preparing 7 μm sections using a Leica CM1950 cryomicrotome (Leica Microsystems, Wttzlar, Germany). After blocking with 1% BSA in PBS, testes sections were incubated with primary antibodies diluted in blocking solution at room temperature for 1 h. After three washes with PBS, testes were labeled with secondary

antibodies for 45 mins. Slides were mounted using VectaShield with 4',6–diamidino–2–phenylindole (DAPI, Vector Laboratories). Images were captured on an LSM710 Zeiss confocal microscope and processed using Adobe Photoshop CS5 software.

The antibodies used were as follows: rat anti-Vasa (DSHB, 1:20); mouse anti-Eya (DSHB, 1:20); mouse anti-FasIII (DSHB, 1:50); rat anti-DE-cadherin (DSHB, 1:20); mouse anti-1B1 (DSHB, 1:75); rabbit anti-Bam C (a gift from DH Chen, 1:2000) (Yang et al., 2007); rabbit anti-Vasa (1:1000, Santa Cruz); rat anti- Zfh-1 (1:5000); rabbit anti-MVH (Abcam, 1:400); goat anti-PLZF *(R&*D, 1:500); and rabbit anti-WT1 (Santa Cruz Biotech, 1:500). Secondary antibodies conjugated to A488, Cy3, A594, or A647 (Molecular Probes and Jackson Immunologicals) were diluted at 1:1000.

Generation of Zfh-1 antibody

cDNA fragments of the *zfh-1* genes were subcloned into the pGEX vector systems to produce GST-fusion proteins containing a fragment of the Zfh-1 proteins (648-775 aa). Generation and affinity–purification of rat polyclonal antisera were conducted as described before (Lai et al., 1991).

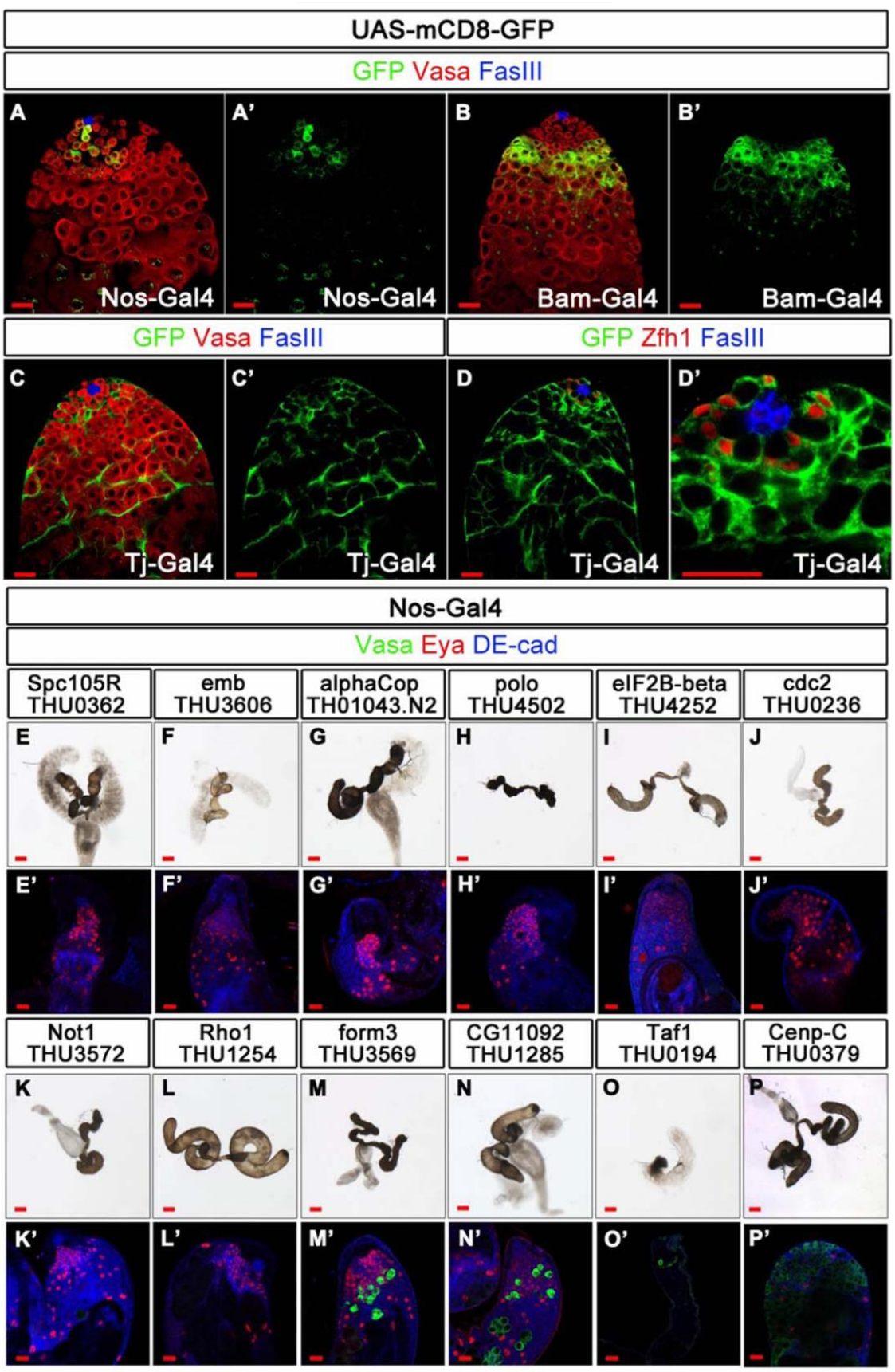

Figure S1. Gal4 expression patterns in the testis and quality controls of the screen. (A–D and A′–D′) The expression patterns of *nos-Gal4* (A and A′), *bam-Gal4* (B and B′) and *tj-Gal4* (C, C′, D and D′) in testes were visualized by crossing to *UAS-mCD8-GFP*. *Nos-Gal4* is mainly expressed in early germ cells including GSCs; *bam-Gal4* is mainly expressed in TA–spermatogonia; *tj-Gal4* is expressed in cyst cells especially in early cyst cells. The testis shown in D' is an enlarged view of the top region in D. (E–P and E′–P′) 12 genes that have two independent RNAi lines show similar phenotypes when both lines were crossed to *nos-Gal4* (data of one RNAi line for each gene were shown). (E–P) Light microscopy view and (E′–P′) confocal view of the testes with indicated gene knockdown. Anti-GFP show the Gal4 expression patterns (green in A–D and A′–D′), Anti-Vasa labels germ cells (red in A–Cand green in E′–P′), anti-FasIII shows the hub cells (blue in A–D and D′), anti-Zfh1 labels the CySCs (red in D and D′), anti-Eya marks the differentiated cyst cells (red in E′–P′), anti-DE-cad labels the cyst cells and hub cells (blue in E′–P′). Scale bars: 100μM for E–P; 20μM for others.

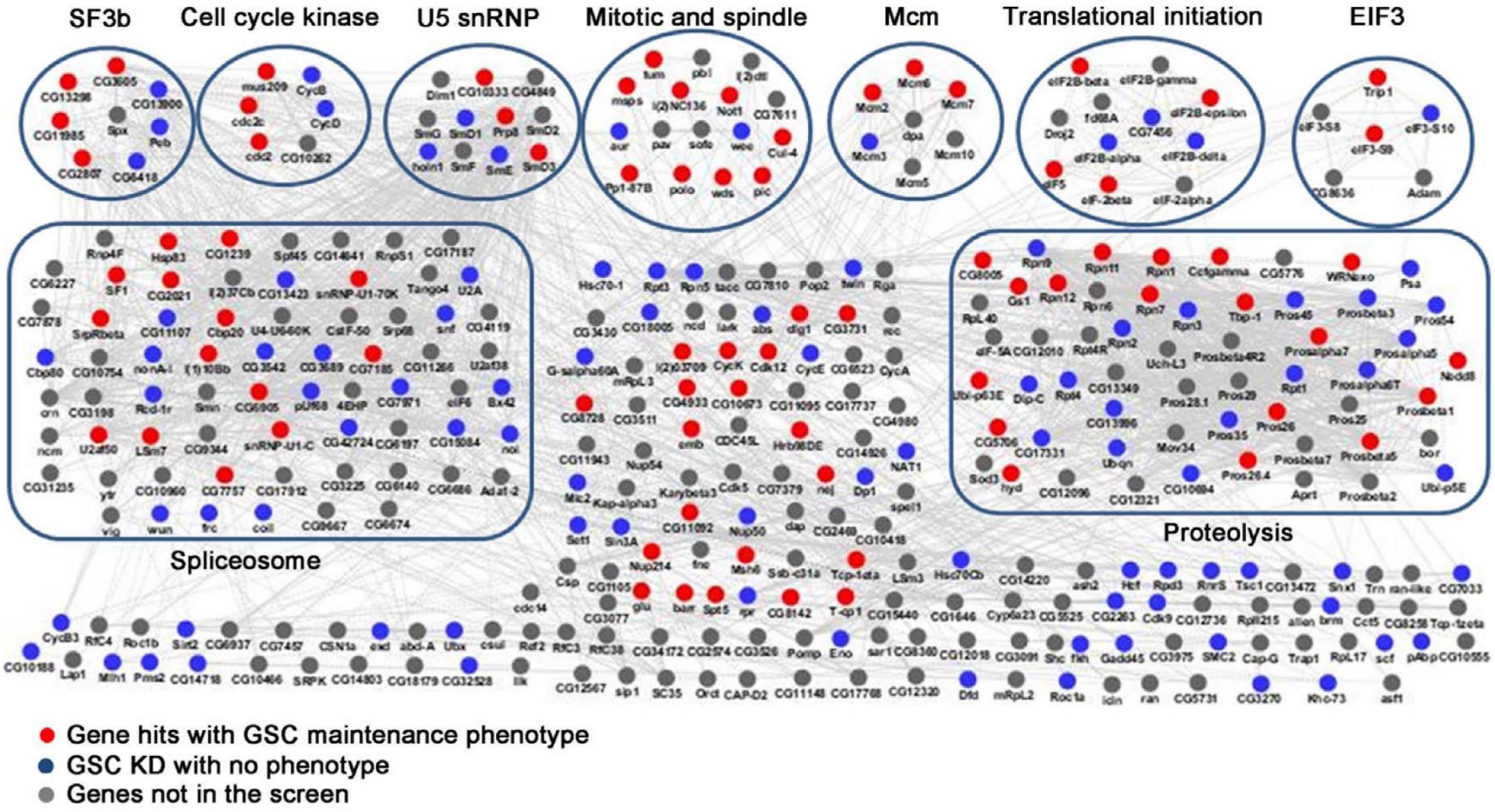

Figure S2. A regulatory network generated by COMPLEAT analysis. Genes are shown as nodes; red nodes indicate genes identified in this screen. Blue nodes are components of complexes not identified in this screen. Gray nodes indicate genes that have not been analyzed in this study. Edges denote interactions between proteins. Solid edges reflect interactions supported by direct experimental evidence from the fly proteins and dotted edges represent putative interactions supported by experimental evidence from the homologous proteins of other species.

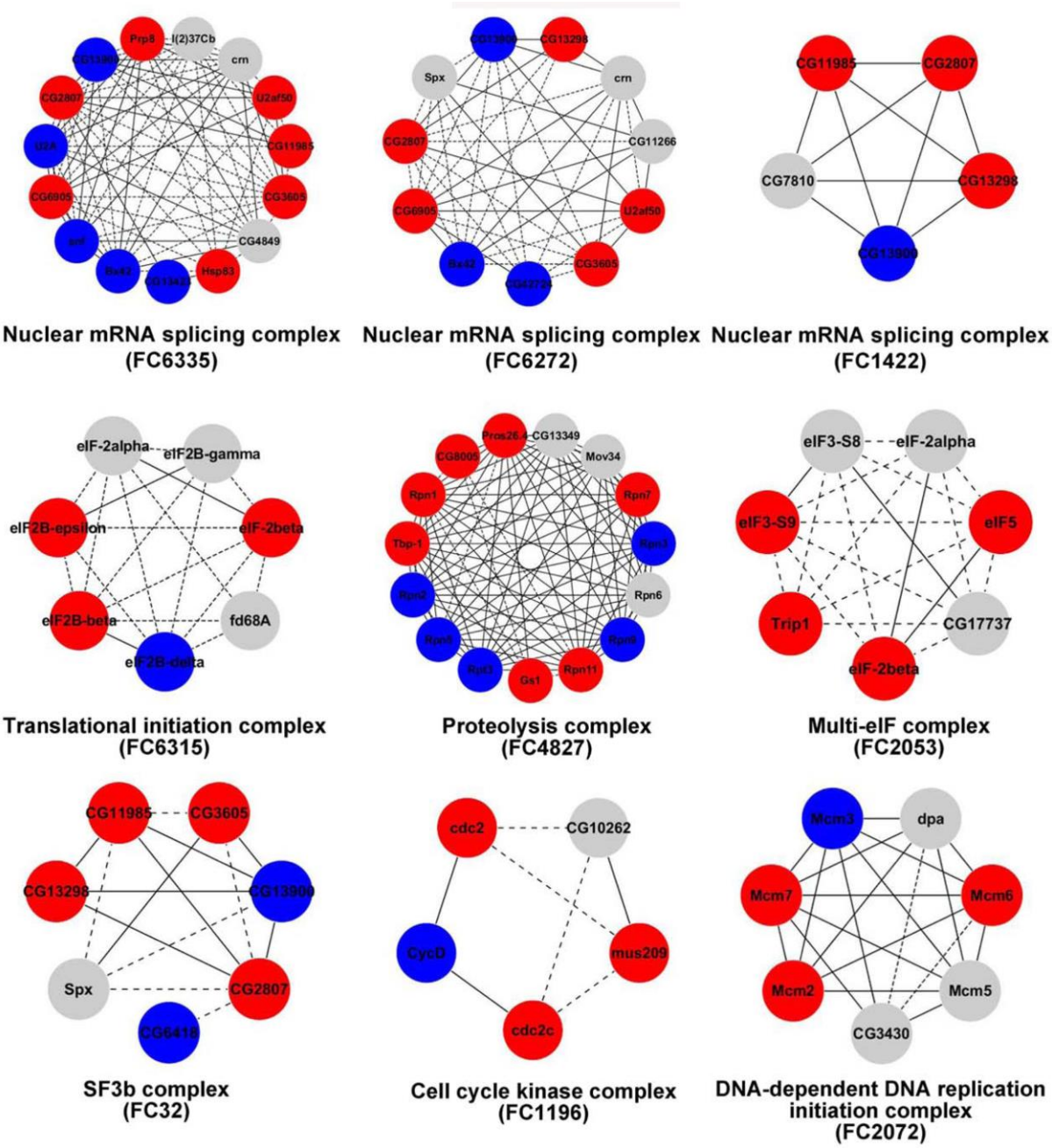

Figure S3. Representative complexes identified in the screen using COMPLEAT analysis. Red nodes indicate genes identified in the screen, blue nodes indicate genes that were not identified in this screen. Gray nodes indicate genes that have not been analyzed in this study. The full list of complex analysis is shown in Table S3.

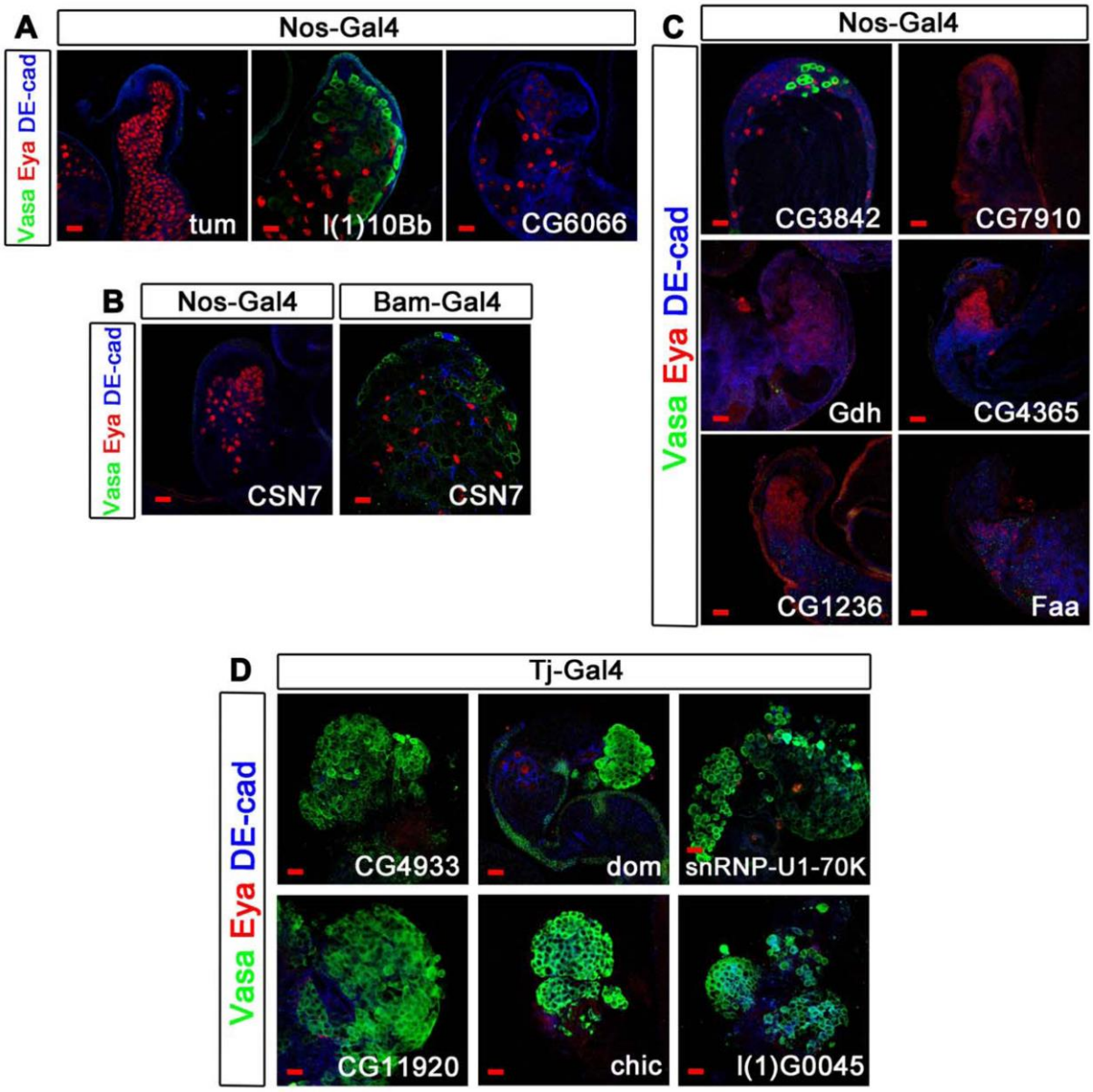

Figure S4. A few RNAi examples mentioned in the main text but did not show in the main figures. The indicated genes were knockdown with listed Gal4s. Anti-Vasa labels germ cells (green), anti-Eya marks the differentiated cyst cells (red), anti-DE-cad labels the cyst cells and hub cells (blue). Scale bars: 20 μM.

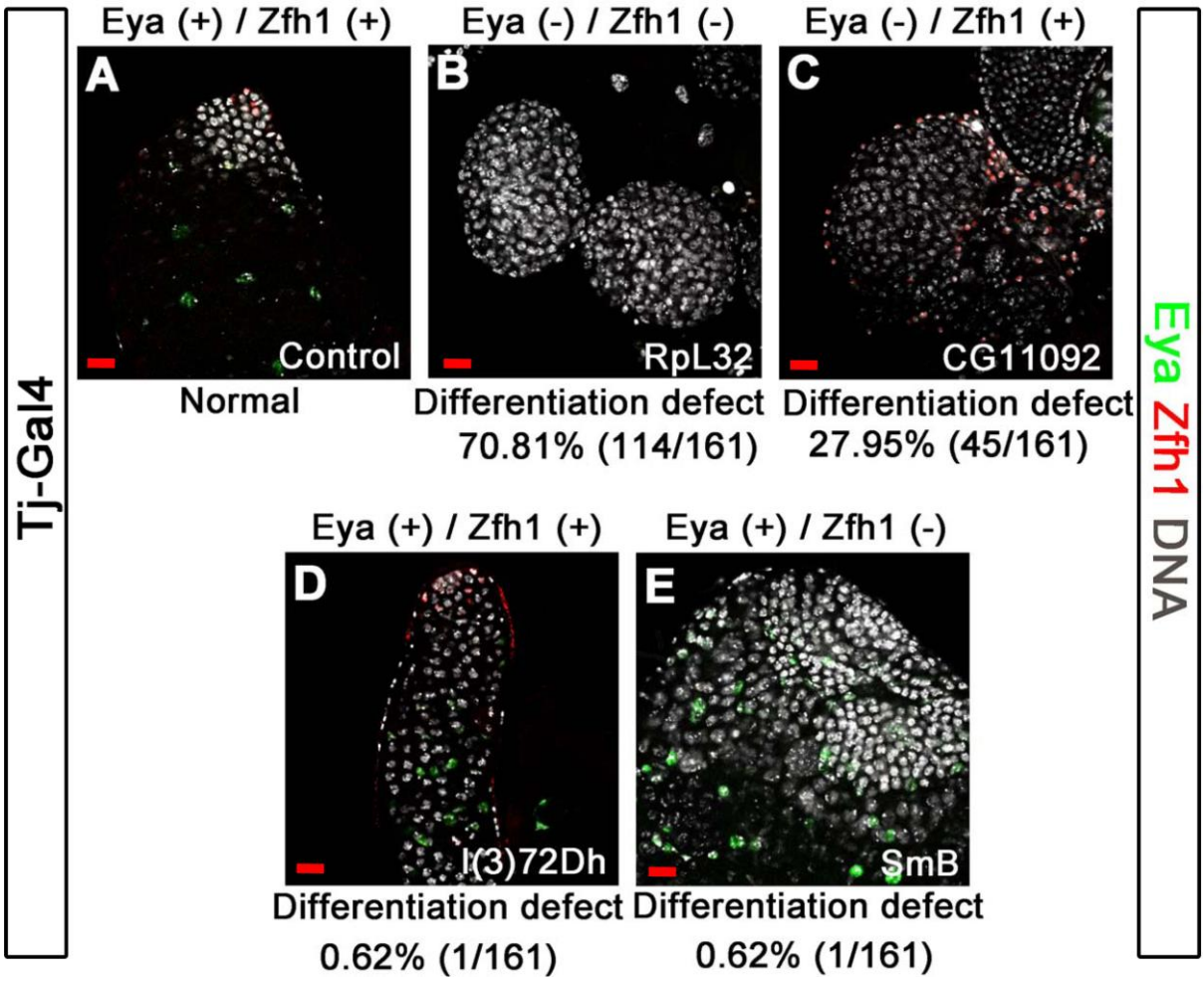

Figure S5. The typical patterns of CysC and cyst cell markers in the testes with differentiation defects resulted from the *tj-Gal4* driven RNAi screening. The indicated genes were knockdown with *tj-Gal4*. Anti-Zfh1 labels CysCs (red), anti-Eya marks the differentiated cyst cells (green), DNA was labeled with Hoechst 33342 (gray). Scale bars: 20 μM. The proportion of the lines that have similar Zfh1 and Eya patterns in the total testes with differentiation defects were labeled under the images. In total, 161 lines show the differentiation defects in the tj-Gal4 screen.

Table S1. Detailed information of the RNAi lines used in this study. Transgenic RNAi lines from *THFC* used for the GSC self-renewal screen.

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Table S2. Phenotype annotation of genes identified in the screen. Sheet1: The detailed phenotypes of the 221 genes required for GSC self-renewal and differentiation. Sheet2: List of 12 genes that have a second independent RNAi line. Phenotypes of the male progeny produced by crossing the second RNAi lines with *nos-Gal4*. Sheet3: Tumor formation frequency and patterns of Eya and Zfh-1 in testes from the *tj-Gal4* screen.

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Table S3. Full list of complexes required for GSC self-renewal and differentiation were generated by COMPLEAT analysis.

Click here to Download Table S3

Table S4. Full list of genes encoding WD40 domain proteins that were identified in this screen.

CG NO.	FlyBase ID	Fly Symbol	Interaction (with pic)	Domains	Human Orthologs	Ensembl Gene ID	Domains	Putative CUL4 CRL Substrate Recepto	Reference (PMID)
CG10080	FBgn0034641	mahj	PPI: DPiM coAP complex	WD40	VPRBP	ENSG00000145041	WD40	Yes	16964240;16949367;23062609;17588513
				WD40	RBBP4	ENSG00000162521	WD40	Yes	17588513
CG4236	FBgn0263979	Caf1	PPI: Predicted from huma	WD40	RBBP7	ENSG00000102054	WD40	Yes	17079684;21228219;17588513
				WD40	WDR26	ENSG00000162923	WD40	Yes	17588513
				WD40	TWF1	ENSG00000151239			
CG17293	FBgn0032030	Wdr82		WD40	TWF2	ENSG00000247596			
				WD40	WDR82	ENSG00000164091	WD40	Yes	17588513
				WD40	TBL3	ENSG00000183751			
CG17437	FBgn0040066	wds	InterologFinder: Predicted	WD40	WDR5	ENSG00000196363	WD40	Yes	17041588;17588513
				WD40	WDR5B	ENSG00000196981	WD40	Yes	17588513
CG5018	FBgn0263605	l(3)72Dn		WD40	CIRH1A	ENSG00000141076	WD40	NO	
CG3820	FBgn0010660	Nup214		WD40	NUP214	ENSG00000126883	WD40	NO	
CG5033	FBgn0028744	CG5033		WD40	BOP1	ENSG00000261236	WD40	NO	
CG6015	FBgn0038927	CG6015		WD40	CDC40	ENSG00000168438	WD40	NO	
CG7961	FBgn0025725	alphaCop		WD40	COPA	ENSG00000122218	WD40	NO	
CG5519	FBgn0261119	Prp19		WD40	PRPF19	ENSG00000110107	WD40	NO	
CG7989	FBgn0262560	wcd		WD40	UTP18	ENSG00000011260	WD40	NO	
CG4878	FBgn0034237	eIF3-S9		WD40	EIF3B	ENSG00000106263	WD40	NO	
CG8882	FBgn0015834	Trip1		WD40	EIF3I	ENSG00000084623	WD40	NO	