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 testis 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
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...
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 testes. 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.

**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 cell population (soma-induced germ cell maintenance defects, Fig. 2L,M) 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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The number of RNAi lines that were generated with indicated vectors used in this study; the number of positive hits with each RNAi line.
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...
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 testes [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.

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.
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.
In the \textit{if-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 \textit{Rpn1}, \textit{Rpn7}, \textit{Rpn11}, \textit{Rpn12}, \textit{Tbp-1}, \textit{Prosaph7}, \textit{Prosbeta1}, \textit{Prosbeta5}, \textit{Pros26} and \textit{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 \textit{if-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 \textit{Zfh-1} and the mature cyst cell marker \textit{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 \textit{Eya} but positive for \textit{Zfh1} (Fig. S5); however, both the number and the distribution of the \textit{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 \textit{Zfh1} and \textit{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 \textit{SmB} with \textit{if-Gal4} results in testes that are positive for \textit{Eya} but negative for \textit{Zfh1} (Fig. S5E), indicating this gene might be crucial for CySC maintenance.

\textit{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 \textit{ptc-Gal4}; \textit{tubGal80}°. Eight genes that have the most severe tumor formation phenotypes were selected (Fig. 5A-D, Fig. 4C and Fig. S4D). \textit{PtC-Gal4}; \textit{tubGal80}°>UAS-RNAi flies were cultured at the permissive temperature until eclosion. We then shifted the culture temperature to 28°C and analyzed the testes at 0, 5, 10 and 20 days. Knockdown of two (\textit{dom} and \textit{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 \textit{ptc-Gal4}; \textit{tubGal80}° 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 \textit{if-Gal4} and the \textit{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 regulating 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 testes 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, \textit{tum}, \textit{l(1)10Bb}, \textit{CG6066} and \textit{eIF5}, were identified in all of our stem cell self-renewal screens (Fig. 1F, cdc2; Fig. 2D and Fig. S4A). \textit{cdc2} and \textit{tum} are genes that are required for cell cycle progression and division (Jin et al., 2005; Jones et al., 2010). \textit{l(1)10Bb} and \textit{CG6066} participate in mRNA splicing (Herold et al., 2009). \textit{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 \textit{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 \textit{nos-Gal4} to drive UAS-shRNA expression leads to loss of germ cells. Interestingly, we also observed early germ cell differentiation defects when \textit{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 \textit{CG3842}, \textit{CG7910}, \textit{Gdh}, \textit{CG4365}, \textit{CG1136} and \textit{Faa}, which regulate GSC self-renewal (Fig. S4C). We also identified multiple genes encoding mitochondrial proteins that were required for GSC maintenance. The protein encoded by \textit{CG7506} regulates assembly of the mitochondrial proton-transporting ATP synthase complex (Cízková et al., 2008). The protein encoded by \textit{CG11722} is required for assembling the mitochondrial respiratory chain complex I (Saada et al., 2008).

**CRL4 E3 ligase complexes are required for GSC self-renewal**

\textit{Nedd8} is intrinsically required for GSC self-renewal in the ovary (Pan et al., 2014). In our screen, knockdown of \textit{Nedd8} using nos
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<sup>ts</sup> led to non-cell autonomous proliferation of undifferentiated germ cells compared with control (E). RNAi of Rpn1 (H) with ptc-Gal4; tub-Gal80<sup>ts</sup> 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.
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) (Fig. 7C), 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 knockout 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 orthologs 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 cysts 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...
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 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 Ddb1fl/fl mice with Ddx4-Cre transgenic mice (Yu et al., 2013). Testes were much smaller in 2-month-old Ddb1fl/−; 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 Ddb1fl/−; 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 cKO 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...
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...
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
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/fl; *Ddx4*-Cre mice testes. (D) Number of SSCs in WT and *Ddb1*fl/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.
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 (Hershey, 2010). 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 Fmr1 (Chen et al., 2014). In *Drosophila*, the adult stage leads to overproliferation of early germ cells (Lim and Fuller, 2012). Tumor formation in *tj-Gal4>*UAS-RNAi testes is mediated by translational control of Mei-P26 (Hershey, 2010). 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 size (small or tiny) of the testis was judged by the length of the testis, which leads to overproliferation of early germ cells (Lim and Fuller, 2012). Tumor formation in *tj-Gal4>*UAS-RNAi testes is mediated by translational control of Mei-P26 (Hershey, 2010). 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.

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): *ros-Gal4* (BDSC, #4937), *tj-Gal4* (DGRC, #104055), *UAS-Der-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: *ptc-Gal4*; *Gal80ts/TM6b* (THFC, #THJ0203). The following mutant alleles were from DGRC and BDSC: *Cul-4*<sup>Δ1612</sup> (DGRC, #111538), *ptc-Gal4*<sup>Δ4937</sup> (BDSC, #26890), *ptc-Gal4*<sup>Δ4937</sup> (BDSC, #15350) and *mah*<sup>Δ1400</sup> (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 leads to overproliferation of early germ cells (Lim and Fuller, 2012). Tumor formation in *tj-Gal4>*UAS-RNAi testes is mediated by translational control of Mei-P26 (Hershey, 2010). 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.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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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


