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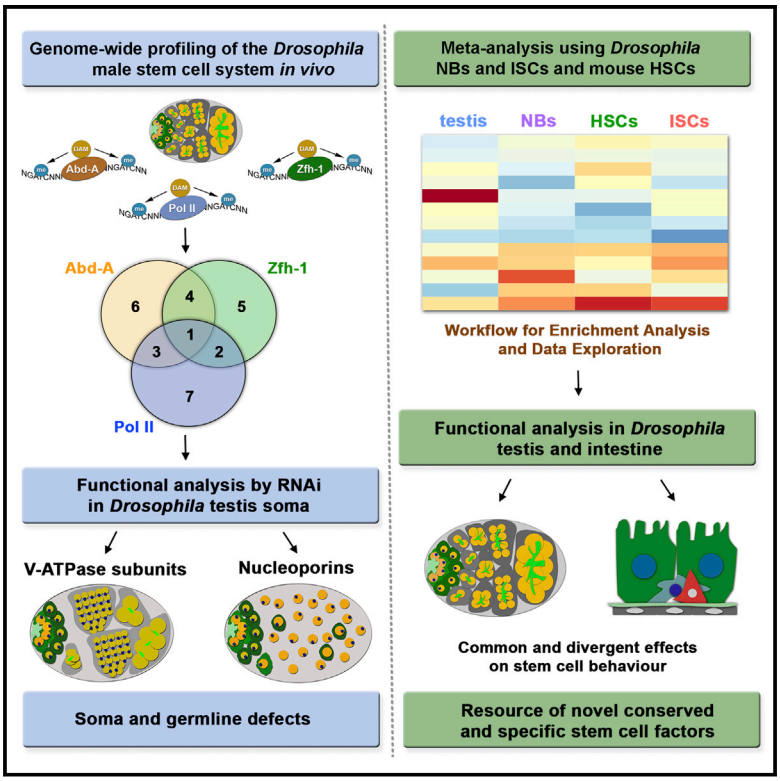
Decoding the Regulatory Logic of the *Drosophila* Male Stem Cell System

Graphical Abstract



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In Brief

Tamirisa et al. performed genome-wide profiling of the *Drosophila* testis to identify stem cell regulators. Comparative analysis using other stem cell systems revealed common and specific stem cell factors. They show that V-ATPase proton pumps and nucleoporins play critical roles in stem cell regulation of the testis.

Highlights

- DamID and TaDa analysis identified stem cell factors in *Drosophila* testis
- Interactive online tool (WEADE) developed for analyzing datasets is publicly available
- Comparative studies identified a high fraction of conserved stem cell regulators
- V-ATPases and nucleoporins are critical for stem cell maintenance

Data and Software Availability

GSE117833

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Decoding the Regulatory Logic of the *Drosophila* Male Stem Cell System

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SUMMARY

The niche critically controls stem cell behavior, but its regulatory input at the whole-genome level is poorly understood. We elucidated transcriptional programs of the somatic and germline lineages in the *Drosophila* testis and genome-wide binding profiles of Zfh-1 and Abd-A expressed in somatic support cells and crucial for fate acquisition of both cell lineages. We identified key roles of nucleoporins and V-ATPase proton pumps and demonstrate their importance in controlling germline development from the support side. To make our dataset publicly available, we generated an interactive analysis tool, which uncovered conserved core genes of adult stem cells across species boundaries. We tested the functional relevance of these genes in the *Drosophila* testis and intestine and found a high frequency of stem cell defects. In summary, our dataset and interactive platform represent versatile tools for identifying gene networks active in diverse stem cell types.

INTRODUCTION

Animal tissues and organs are generated and maintained by adult stem cells, which remain undifferentiated and proliferative while at the same time producing daughter cells that undergo differentiation. Due to this ability, they can replace dying, lost, or damaged cells and are thus critical for tissue homeostasis. The balance between self-renewal and differentiation is tightly controlled, and stem cell-intrinsic mechanisms are known to play an important role in this process (Biteau et al., 2011; Pearson and Sánchez Alvarado, 2008). In addition, signals from the stem cell niche are equally important to control the activity of stem cells and their progeny (Hsu and Fuchs, 2012; Morrison and Spradling, 2008). However, it is still poorly understood how niche cells execute their regulatory function on a global level.

The *Drosophila* testis represents an excellent model for studying stem cell-niche interactions (de Cuevas and Matunis, 2011). At the tip of the testis, germline stem cells (GSCs) and somatic cyst stem cells (CySCs) maintain spermatogenesis (Fuller and Spradling, 2007). Each GSC is enclosed by two CySCs, and both stem cells are anchored to nondividing somatic cells, called the hub (Hardy et al., 1979), through adhesion molecules (Matunis et al., 1997; Wang et al., 2006). GSCs and CySCs divide asymmetrically to produce gonialblasts (Gbs) and somatic cyst cells (SCCs) that form a developmental unit called cyst. Whereas gonialblasts undergo four rounds of mitotic divisions to produce 16 spermatogonia that develop into spermatocytes, SCCs grow without division and co-differentiate with the germline (Figure 1A) (Fuller and Spradling, 2007).

The somatic cell population fulfills several support functions in the *Drosophila* testis. Hub cells express Unpaired (Upd), the ligand that activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway in adjacent CySCs, instructing their maintenance (Kiger et al., 2001; Tulina and Matunis, 2001) and regulating GSC anchoring to the hub (Leatherman and Dinardo, 2008; Stine et al., 2014). CySCs and their progenies are equally critical for germline development. They not only induce GSC fate by signaling to germ cells via the transforming growth factor-β (TGF-β) pathway (Shivdasani and Ingham, 2003) but also instruct the self-renewal of GSCs. The transcriptional regulator zinc-finger homeodomain protein 1 (Zfh-1), which is activated by JAK-STAT signaling in CySCs, is key for the soma-germline crosstalk and CySC development (Leatherman and Dinardo, 2008). Importantly, Zfh-1 is sufficient for the induction of GSC self-renewal, even outside the niche (Leatherman and Dinardo, 2008), and controls an uncharacterized gene network essential for soma and GSC maintenance. Moreover, the close communication between somatic and germline cells is required not only for GSC maintenance but also for later stages of spermatogenesis (Fabrizio et al., 2003; Kiger et al., 2001). In sum, these studies highlight the importance

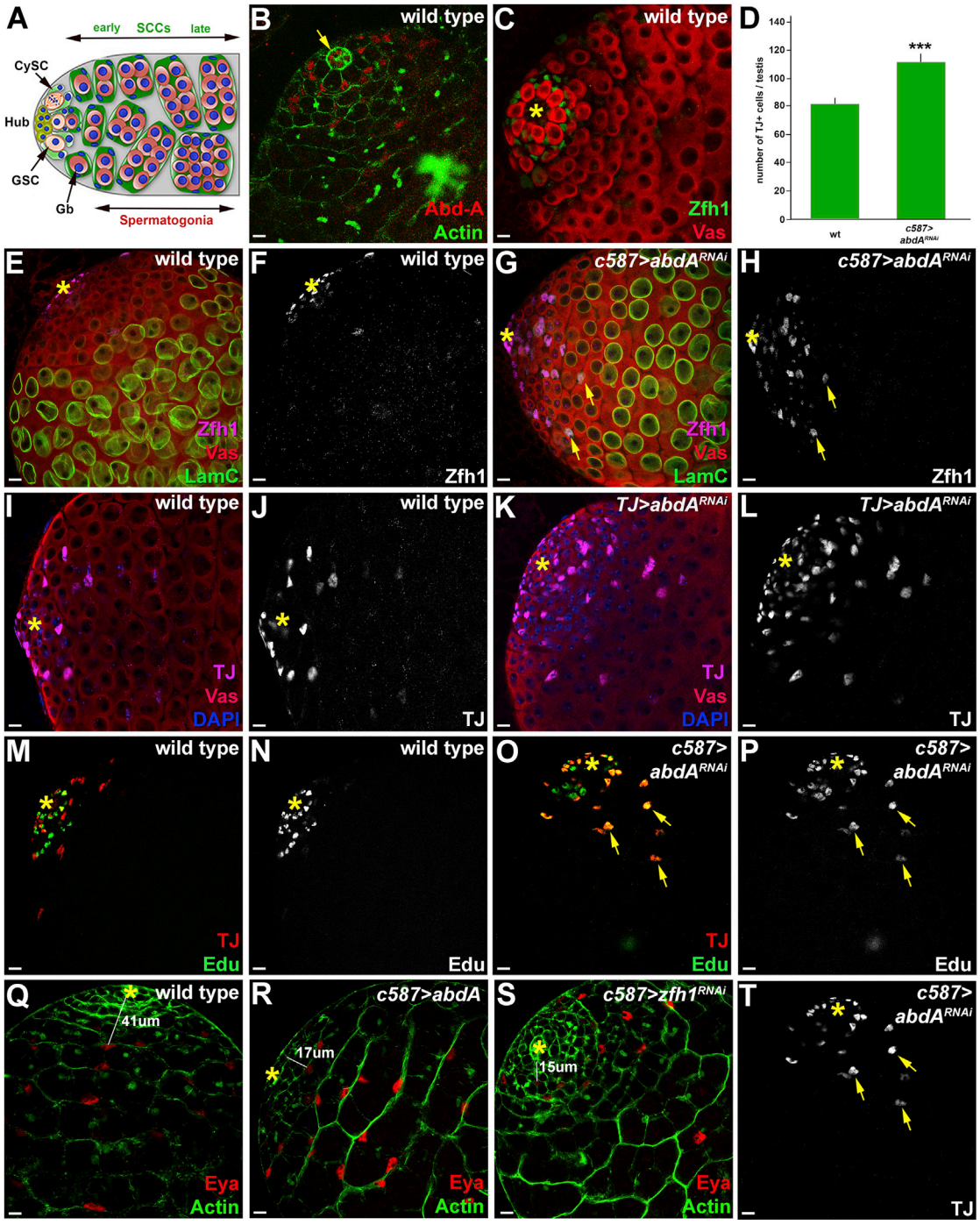

Figure 1. The Hox Transcription Factor Abd-A Controls the Switch from CySCs to Early and Late SCCs

(A) Schematic diagram of early spermatogenesis in *Drosophila* third-instar larval (L3) testis. CySC, somatic cyst stem cell; Gb, gonialblast; GSC, germline stem cell; SCCs, somatic cyst cells.

(B) L3 wild-type testes stained for Abd-A (red) and actin (green) to mark cyst cells and germline fusomes. The yellow arrow marks the hub.

(C) L3 wild-type testis stained for Zfh-1 (green) and Vasa (red).

(D). Quantification of TJ-positive cells in wild-type and *c587 > abdA*^{RNAi} testes (***p < 0.001).

(E–H) L3 wild-type (E and F) and *c587 > abdA*^{RNAi} (G and H) testes stained for Zfh-1 (magenta), Vasa (red), and LamC (green). Yellow arrows point to Zfh-1-positive cells in the vicinity of LamC-labeled spermatocytes.

(I–L) L3 wild-type (I and J) and *TJ > abdA*^{RNAi} (K and L) testes stained for TJ (magenta), Vasa (red), and DAPI (blue) for DNA.

(legend continued on next page)

of the soma for the activity of the niche and stem cells, as well as for the maintenance of the testis.

Here, we applied genome-wide *in vivo* mapping of DNA-protein interactions to determine genes active in the *Drosophila* larval testis soma and germline as well as genes targeted by two transcription factors (TFs) active in the somatic lineage. One of them is the well-described stem cell regulator Zfh-1 (Leatherman and Dinardo, 2008), and the other one is the Hox TF Abdominal-A (Abd-A), for which we uncovered a stem cell function in the *Drosophila* testis. Meta-analysis of these datasets recovered a set of genes known to control stem cell development in the *Drosophila* testis and identified a large number of regulators. To make our dataset accessible and amenable to comparative analysis, we developed an interactive data-mining and analysis tool. Data cross-comparison using this tool allowed us to identify major processes active in the soma. By comparing our data to published datasets from diverse stem cell systems and organisms, we uncovered a core gene set of adult stem cells conserved across species boundaries. Testing TFs from this category revealed an unusually high frequency of stem cell phenotypes in the *Drosophila* testis and intestine. Moreover, our analysis identified system specific regulators, which may represent factors that allow individual cell types to respond to and interact with their typical yet diverse microenvironments. Taken together, we elucidate mechanisms used by somatic support cells to control the activity of neighboring germ cells and provide a rich resource to identify important (and conserved) regulators of the proliferation-differentiation balance in a genetically tractable stem cell system.

RESULTS

Defining Abd-A as a Regulator in the *Drosophila* Testis Soma

To uncover gene activities in the testis soma, we determined the transcriptome of the somatic and germline lineages by RNA polymerase II Targeted DamID (TaDa) (Southall et al., 2013), followed by the identification of genes bound by two regulators active in somatic subpopulations and controlling their development using regular DNA adenine methyltransferase identification (DamID) (van Steensel et al., 2001). By combinatorial analysis of these datasets, we expected to identify genes specifically active in *Drosophila* testis support cells and contributing to their functions.

We focused on Zfh-1, which is highly expressed in CySCs (Figures 1C, 1E, 1F, 2A, and 2A′) and known to block CySCs differentiation as well as to control GSC self-renewal (Leatherman and Dinardo, 2008). However, Zfh-1 has no known role in early SCCs, which support the first stages of germline differentiation. To elucidate genes regulating these events within early SCCs, we aimed at identifying a TF controlling early SSC behavior by screening our transcriptome datasets for TFs with exclusive expression in the soma. We excluded Traffic Jam (TJ) and Chinmo, two factors known to be active in CySCs and early SCCs (Flaherty et al., 2010; Li et al., 2003), as TJ is particularly critical for hub specification (Wingert and DiNardo, 2015), while Chinmo is also expressed in germ cells (Flaherty et al., 2010). One candidate was the Hox TF Abd-A, and expression analysis revealed Abd-A accumulation in hub cells, CySCs, and early SCCs of third-instar larval (L3) testes (Figure 1B), similar to the CySC and early SCC marker TJ (Figures 1I and 1J). While Abd-A and Zfh-1 expression partially overlaps (Figures 1B and 1C), they have clearly distinct functions in the testis. Cell-type-specific knockdown of *abd-A* using the *c587*-GAL4 driver (Figure 3A) (Manseau et al., 1997) resulted in testes strongly expressing Zfh-1 not only in CySCs but also in SCCs far away from the hub (Figures 2A–2B′), leading to increased numbers of SCCs co-expressing Zfh-1 and the late SCC marker Eya (Figure 2C). Thus, somatic cells with sustained Zfh-1 expression formed cysts with early spermatocytes marked by Lamin C (LamC) (Figures 1E–1H). This result indicated that Abd-A controlled SCC differentiation by mediating the transition from the CySC to SCC fate. Consistently, TJ-positive cells were expanded when *abd-A* was depleted in the soma using the *c587*-GAL4 or *TJ*-GAL4 lines (Tanentzapf et al., 2007) (Figures 1I–1O and 1T). Conversely, overexpression of *abd-A* resulted in fewer Zfh-1 positive cells (data not shown), as well as an expansion of Eya to the anterior, leading to Eya-positive cells close to the hub (Figure 1R), which was never the case in wild-type testes (Figure 1Q) (Fabrizio et al., 2003). Indeed, the distance between the hub and Eya-positive cells was decreased in *c578>abdA* as well as in *c587 > zfh1*^{RNAi} testes (Figures 1Q–1S), indicating that cells close to the hub started to differentiate when Abd-A was overexpressed (or Zfh-1 activity was reduced). This was not the case when Abd-A was ectopically expressed in late SCCs using the *eyes absent (eya)* GAL4 driver (Leatherman and Dinardo, 2008) (Figures 2D and 2E), showing that CySCs and early but not late SCCs responded to Abd-A expression. By performing Edu 5-ethynyl-2′-deoxyuridine (EdU) incorporation assays, we found TJ-expressing and Edu-positive cells located several cell diameters away from the hub in *abd-A* knockdown testes (Figures 1O, 1P, and 1T), which we never observed in wild-type testes (Figures 1M and 1N). Defects in the soma were not due to Abd-A's expression in the hub (Figure 1B), as neither soma-specific knockdown nor overexpression of Abd-A affected morphology or size of the Fasciclin 3 (Fas3)-stained hub (Figures 2L–2N). To test whether Abd-A controls the JAK-STAT and Hedgehog pathways, which are known to control CySC fate and behavior (Kiger et al., 2001; Michel et al., 2012; Tulina and Matunis, 2001), we performed immunostainings and found that they were unaffected in *abd-A* knockdown (Figures S1A–S1D and S2A –S2D) and gain-of-function conditions (Figures S1A, S1B, S1E, and S1F). In sum, these results showed that Abd-A regulates the identity of early and late SCCs, Abd-A levels are critical for the switch from CySC to early SCC and then to late SCC fate,

(M–P and T) L3 wild-type (M and N) and *c587 > abdA*^{RNAi} (O, P, and T) stained for TJ (red) and Edu (green). Overproliferating SCCs marked by TJ (O and T) and Edu (O and P) are shown by yellow arrows.

(Q–S) L3 wild-type (Q), *c587>abdA* (R), and *c587 > zfh1*^{RNAi} (S) testes stained for Eya (red) and actin (green). White lines indicate the distances between the hub and first Eya-positive cells in the testes. Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S1.

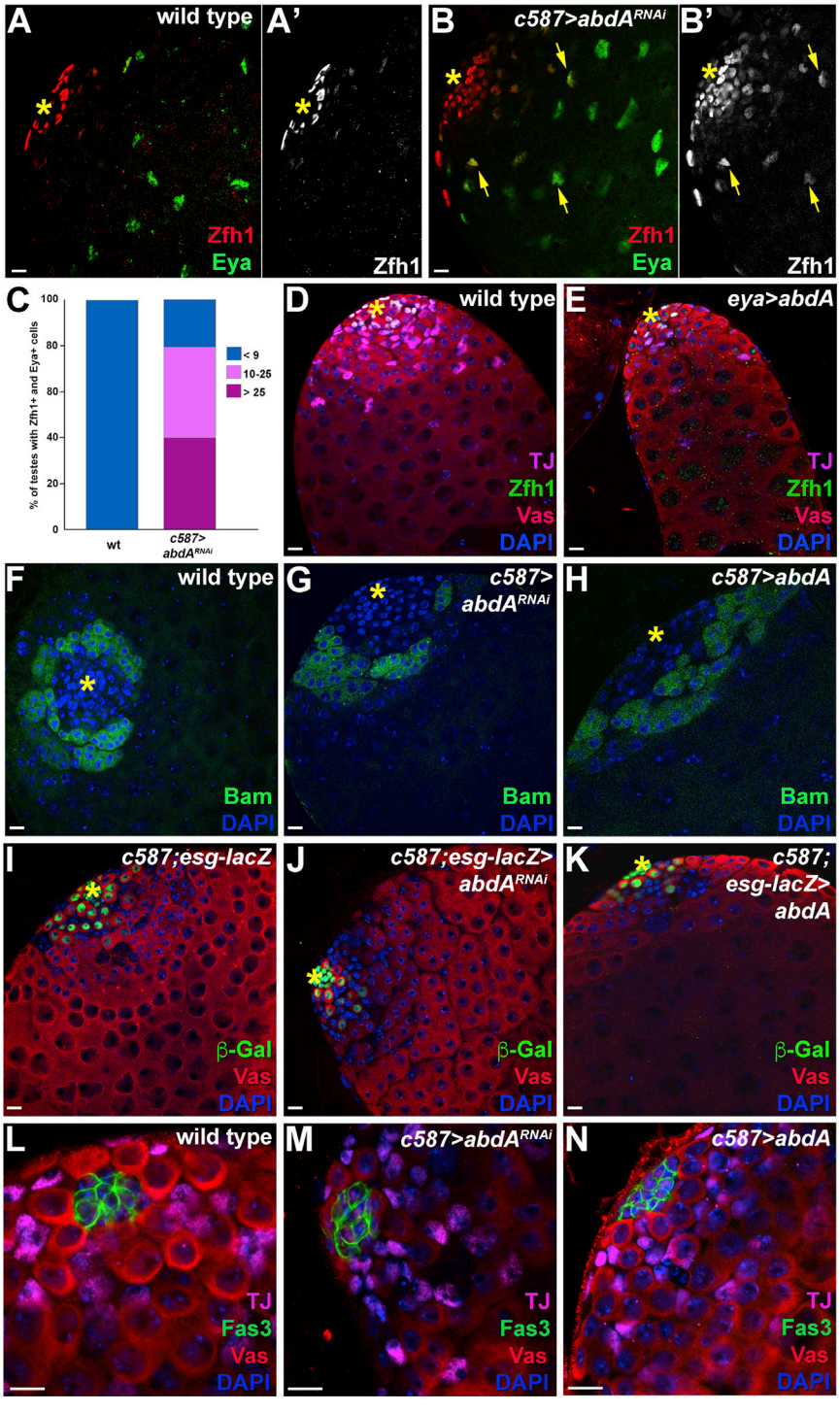

Figure 2. Abd-A Does Not Control Development of the Germline

(A–B′) L3 wild-type (A and A′) and *c587 > abdA*^{RNAi} (B and B′) testes stained for Zfh-1 (red) to label CySCs and with the late SCC marker Eya (green). Knockdown of *abd-A* results in co-localization of Zfh-1- and Eya-expressing cells far away from the hub (marked by yellow arrows in B and B′).

(C) Quantification of the number of Zfh-1 and Eya double-positive cells in wild-type and *c587 > abdA*^{RNAi} testes.

(D and E) Wild-type (D) and *eya>abdA* (E) adult testes stained for TJ (magenta), Zfh-1 (green), Vasa (red), and DAPI (blue) for DNA.

(F–H) L3 wild-type (F), *c587 > abdA*^{RNAi} (G), and *c587>abdA* (H) stained for the germline differentiation marker Bam (green) and DAPI (blue) for DNA.

(I–K) L3 *c587-GAL4;esg-lacZ* (I), *c587;esg-lacZ > abdA*^{RNAi} (J), and *c587;esg-lacZ > abdA* (K) testes stained for β-Gal (green), Vasa (red), and DAPI (blue).

(L–N) L3 wild-type (L), *c587 > abd-A*^{RNAi} (M), and *c587>abdA* (N) stained for Fas3 (green), TJ (magenta), Vasa (red) and DAPI (blue). Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S2.

and Abd-A negatively controls proliferation of somatic cells when they enter the differentiation program.

To elucidate whether Abd-A, similar to Zfh-1 (Leatherman and Dinardo, 2008), controlled germline development, we investigated germline subpopulations in *abd-A* knockdown and overexpression settings, GSCs and gonialblasts using the *escargot (esg)-lacZ* reporter (Gönczy and DiNardo, 1996), and differentiating germ cells using the differentiation marker Bam. This analysis revealed that neither germline maintenance nor differentiation was affected, as both markers were unchanged (Figures 2F–2K).

In sum, our analysis showed that Zfh-1 and Abd-A represent ideal candidates to identify genes controlling processes active in the early stages of the somatic support cells and critical for non-autonomously regulating the balance between stem cell self-renewal and differentiation in the germline.

A Functional Gene Expression Atlas of the *Drosophila* Testis Soma

In a next step, we identified genes expressed in the *Drosophila* testis soma and germline by driving a fusion protein consisting of the *Escherichia coli* Dam and Pol II (UAS-*Dam-PolII*) either throughout the somatic cell population using the *c587*-GAL4 driver (Figure 3A) or the germline lineage using the *nanos (nos)*-GAL4 line (Figure S3A) (Van Doren et al., 1998). We also mapped genome-wide binding profiles of Zfh-1 and Abd-A by generating Dam-Abd-A and Dam-Zfh-1 transgenic flies and using leaky expression from the heat-shock promoter (Tolhuis et al., 2011; van Steensel et al., 2001).

We relied on the DamID strategy, because it had been successfully used in the past for mapping binding sites of TFs active

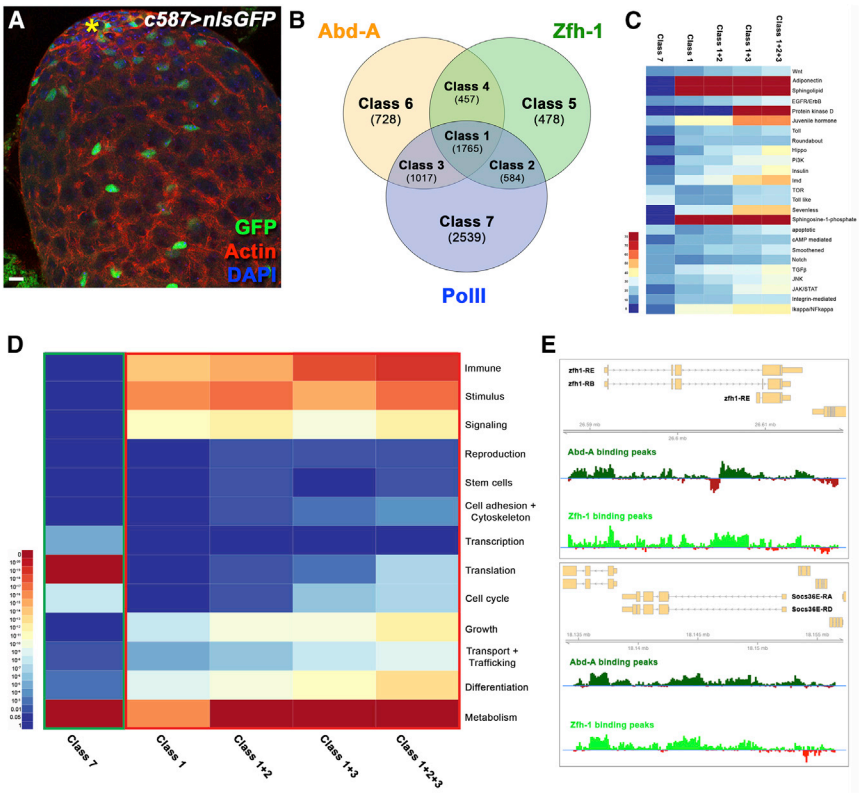

Figure 3. Profiling and Comprehensive Analysis of the *Drosophila* Testis Soma

(A) *c587 > nlsGFP* L3 testis stained for GFP, actin to mark cyst cells, and DAPI for DNA. The yellow asterisk marks the location of the hub.

(B) Venn diagram showing the overlap of genes bound by Zfh-1 (green) and Abd-A (orange) and expressed in the soma (PolII) (blue) in *Drosophila* L3 testes. Gene numbers in the different gene classes are indicated.

(C) Heatmap displaying presence of genes belonging to different signaling pathways in five major gene classes. The color range corresponds to the fraction of genes annotated to the category that also appear in the sample: dark red, 75% of the genes of the category are present in the sample; dark blue, none of the genes in the category are present in the sample.

(D) Heatmap displaying enrichment of general higher-order categories. The color represents the p values: dark red, $<10^{-20}$; dark blue, >0.05. Green frame highlights all genes active, but not bound by Zfh-1 and Abd-A (class 7); red frame highlights all gene classes containing active genes targeted by Zfh-1 and/or Abd-A.

(E) Zfh-1 and Abd-A-Dam binding profiles for the *zfh-1* and *Socs36E* loci. Average peak intensity calculated from 2 repeats in dark and light green, respectively. Associated genes or transcript are shown in light orange.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S3.

in stem cells (Jin et al., 2015) and allows to identify binding events over the course of testis development. In contrast, other genomic approaches, such as RNA sequencing (RNA-seq) or chromatin immunoprecipitation sequencing (ChIP-seq), can only provide snapshots of individual time points. Expression of the Dam fusion proteins had no visible or molecular effect on testis development; fully functional adult testes were formed, which showed gene expression indistinguishable from wild-type testes (Figures S3C–S3H). Methylated DNA was purified and detected by hybridization to *Drosophila* tiling arrays, a standard high-throughput technology for transcriptome profiling with similar output performance as RNA sequencing (Chen et al., 2017; Kogenaru et al., 2012). Enriched binding regions were defined by comparing PolII/Zfh-1/Abd-A methylation profiles to a Dam-alone control (see Experimental; Procedures). We called genes that had at least one enriched genomic region within 2 kb of the gene body in both DamID replicates as targets. For gene detection in TaDa experiments, we used high stringency parameters (FDR < 0.01), which led to the recovery of 5,905 genes expressed in larval somatic cells (Figures 3B and S3B). This list included *zfh-1* and *abd-A* as well as many other genes known to be active in the testis soma (Table S3). For the germline, we identified 2,199 expressed genes using the same strategy (Figure S3B).

The Dam-fusion proteins were equally expressed in the soma and germline, while endogenous Zfh-1 and Abd-A are exclusively active in the soma. Thus, we analyzed our datasets for specificity by comparing active genes identified by germline or soma TaDa with the 2,686 genes that are targeted by Abd-A and/or Zfh-1. We found only 6% (222/2,686) of the germline-restricted active genes to be associated with Abd-A and/or Zfh-1 binding events, while 94% (2,464/2,686) of the genes expressed exclusively in the soma were targeted by either or both TFs (Figure S3B). This result demonstrated that Zfh-1 and Abd-A interacted with *cis*-regulatory regions preferentially in the somatic lineage, maybe due to permissive chromatin environments or availability of suitable cofactors.

After having confirmed the specificity of our datasets, we excluded the germline-specifically expressed and TF-targeted genes from the analyses and combined the soma-specific transcriptome with the TF-binding datasets. Among the seven gene classes identified (Figure 3B; Table S1), we considered the somatically expressed genes bound exclusively by Zfh-1 (class 2) or Abd-A (class 3) or by both TFs (class 1) (3,366 genes in total) (Figure 3B) to contain many genes expressed in CySCs and early SCCs in larval testis development (Table S1). Consistently, 15 genes known to be active in CySCs and early SCCs were present in these three classes (Table S3; Figures 3E, S3I, and S3J). Genes targeted by both TFs (class 4) or exclusively by Zfh-1 (class 5) or Abd-A (class 6), but not recovered in the PolII TaDa experiment (1,663 in total) (Figure 3B), were classified as genes inactive in the testis soma and either actively repressed by Zfh-1 and/or Abd-A or neutrally bound (Table S1). And finally, genes recovered in the soma-specific PolII TaDa experiment but not associated with Zfh-1 or Abd-A binding (2,539) (class 7) (Figure 3B) were categorized as genes expressed throughout the soma, with their expression being independent of Zfh-1 and Abd-A inputs (Table S1). Importantly, 75% of somatically

expressed genes targeted by Zfh-1 were co-bound by Abd-A (Figure 3B), supporting the important function of Abd-A in the soma and suggesting that the two TFs converge on a similar stem cell-related output program.

Gene Functions Active in the Support Cells of the *Drosophila* Testis

To obtain functional insights into the gene classes, we performed standard gene ontology (GO) and pathway analyses (Cabezas-Wallscheid et al., 2014; Dutta et al., 2015; Llorens-Bobadilla et al., 2015). In addition, we developed a freely available online tool to elucidate diverse and common signatures in the different gene classes by assessing the enrichment of functionally related GO terms combined into higher-order categories (Table S4) in a comparative fashion across multiple samples (see Experimental Procedures for details) (Trost et al., 2018).

We first tested our assignment of gene activities to somatic cell populations (Table S1). To this end, we compared processes overrepresented in the Zfh-1 and Abd-A active class (classes 1, 1+2, 1+3, 1+2+3), which we assumed to be required in CySCs and early SCCs (Table S1), with those enriched in the control class (class 7), which we defined as essential processes in all somatic cells not controlled by Abd-A and Zfh-1 (Table S1). This analysis revealed a striking correlation of functional categories with the diverse cell types in the somatic lineage and known requirements for stem cell regulatory processes. For example, the functional term "Translation" was significantly overrepresented among genes of class 7, while this term was only weakly represented in the classes defined by Zfh-1 and Abd-A binding (Figure 3D). This finding is consistent with the low translational rates recently described for diverse stem cell types (Slaidina and Lehmann, 2014; Llorens-Bobadilla et al., 2015; Signer et al., 2014). Conversely, we found differentiation-related genes to be prominently overrepresented in Zfh-1 and Abd-A active classes, but not in class 7 (Figure 3D). Thus, differentiation processes seem to be primed in CySCs (and early SCCs), which is in line with functional studies in the testis (Figures 1E–1T) (Leatherman and Dinardo, 2008), and included the TF-encoding genes *Stat92E* (Kiger et al., 2001), *zfh-1*, and *CtBP* (Leatherman and Dinardo, 2008). In addition, our tool recovered many other differentiation genes so far not known to control development of the testis soma, including the TFs myeloid leukemia factor (Mlf), Hyrax (Hyx), and female sterile (1) homeotic (Fs(1)h).

Signaling and stimulus terms were also enriched among the genes of the Zfh-1 and Abd-A active classes, but not in class 7 (Figure 3D). This finding may reflect the well-known dependency of the male stem cell niche on the proper interplay of signaling pathways (Kiger et al., 2001; Sinden et al., 2012). Intriguingly, components of the JAK-STAT (Kiger et al., 2001; Tulina and Matunis, 2001), epidermal growth factor receptor (EGFR) (Chen et al., 2013a), Hedgehog (Michel et al., 2012), mitogen-activated protein kinase (MAPK) (Amoyel et al., 2016a), TGF-β (Li et al., 2007), Hippo (Amoyel et al., 2014), and phosphatidylinositol 3-kinase (PI3K)/Tor (Amoyel et al., 2016b) pathways, which had been shown to be active and functional in somatic support cells, were highly enriched in Zfh-1 and Abd-A active classes but much less in class 7 (Figure 3C). In addition, we identified signaling pathways so far not well studied in the *Drosophila* testis soma, including the Wnt, Toll, and juvenile hormone pathways (Figure 3C). These findings now open new avenues to resolve the individual and combinatorial contribution of these signaling pathways in balancing stem cell maintenance and differentiation.

A Comparative Approach Identifies a High Fraction of Stem Cell Regulators

To select among the large number of genes the most significant ones for functional studies, we took a comparative approach by intersecting our data with published expression data retrieved from other stem cell systems. Our reasoning was that genes expressed in multiple stem cell types should regulate stem cell behavior with a higher probability. We selected data from *Drosophila* neural stem cells (NBs) (Southall et al., 2013) and intestinal stem cells (ISCs) (Dutta et al., 2015), as well as mammalian hematopoietic stem cells (HSCs), to delineate common and divergent genes and processes across functionally but also evolutionary diverse stem cell systems. These datasets were generated using diverse technologies, cell sorting, and RNA-seq for ISCs (Dutta et al., 2015) and HSCs (Cabezas-Wallscheid et al., 2014), while NBs (Southall et al., 2013) and the testis soma were profiled using DamID. For NBs, the list of genes defined as active was taken from the original publication (Southall et al., 2013); for the testis soma, we used the Abd-A and Zfh-1 active genes (class 1+2+3); and for ISCs and HSCs, the top 50% of detected transcripts were analyzed.

We first performed GO term analysis using our tool, which identified a small number of processes to be overrepresented in all systems, in particular cell growth and cell cycle (Figure 4B). Processes related to cytoskeleton and cell adhesion as well as signaling were found moderately overrepresented (Figure 4B), which is in line with the intimate stem cell-niche interaction and communication critical for long-term maintenance and function of stem cell systems (Chen et al., 2013b). Importantly, Wnt, MAPK, JAK/STAT, EGFR, and the Hippo pathways were significantly overrepresented in all four stem cell systems (Figure S4T). Several signatures were different in the various stem cell systems, including a high representation of stem cell-related processes in NBs, metabolic processes in HSCs, and the immune response in the testis soma (Figure 4B).

We next searched for individual genes common to all datasets; thus, we used orthology mapping to convert mouse gene identifiers into *Drosophila* gene identifiers. Intersecting whole-transcriptome profiling of fluorescence-activated cell-sorted (FACS) mouse HSCs (Cabezas-Wallscheid et al., 2014) with the *Drosophila* datasets identified 716 transcripts common to all systems (Figure 4A), with 21 of them encoding TFs (Tables S2 and S5). TFs had been demonstrated to control the balance between self-renewal and differentiation in diverse stem cell systems (Takahashi and Yamanaka, 2006; Yamanaka and Blau, 2010), and consistently, some of the TFs had already reported functions in different stem cell types. (Leatherman and Dinardo, 2008; Zhu et al., 2011; Dominado et al., 2016; Viatour et al., 2008). We next tested the majority of TF encoding genes in the *Drosophila* testis, revealing that 7 out 17 (41%) of the commonly expressed TFs controlled testis development (Table S5). Importantly, knockdown of these TFs resulted in diverse phenotypes, showing that they fulfill distinct regulatory roles. For example,

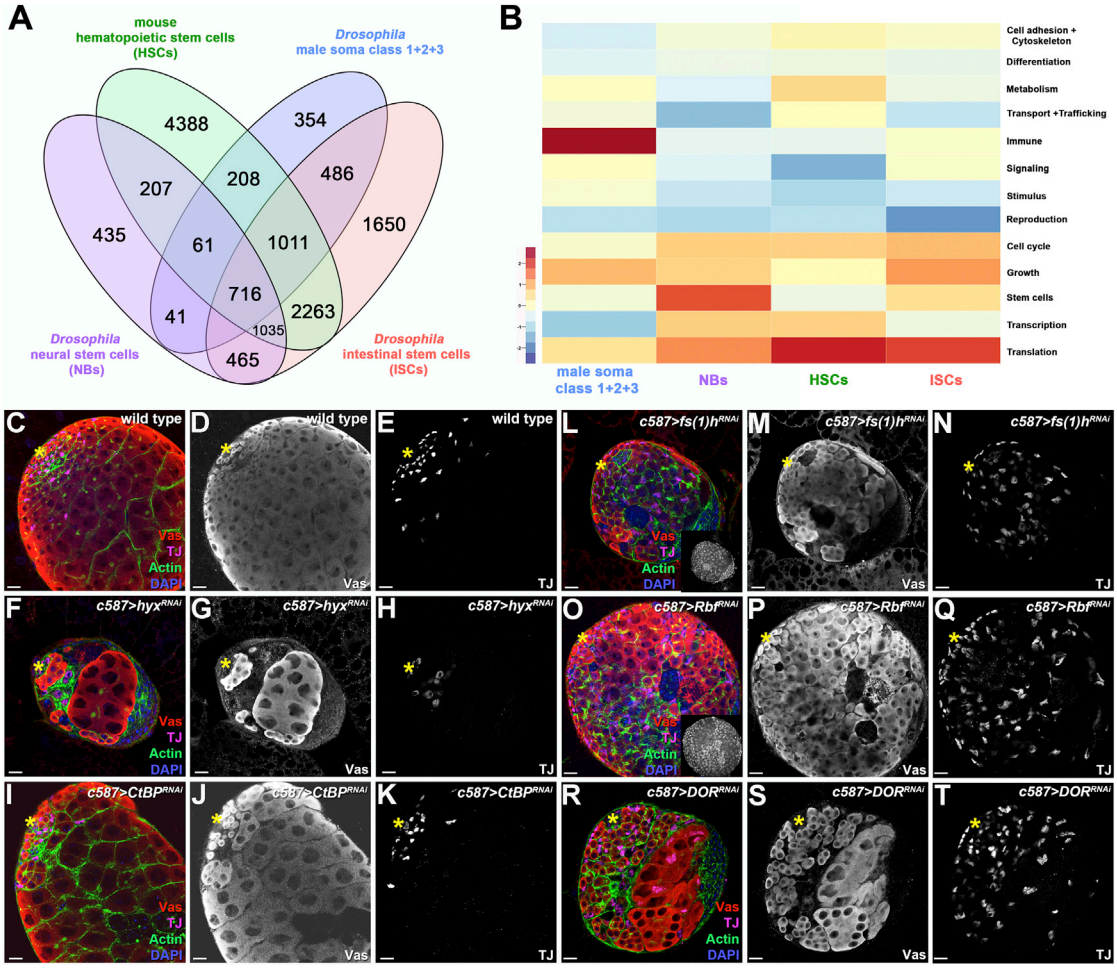

Figure 4. Comparative Analysis of Different Stem Cell Systems Identifies Commonly Expressed TFs with Functional Relevance in the *Drosophila* Testis

(A) Comparison of the transcriptomes of *Drosophila* neural stem cells (NBs) (purple), *Drosophila* intestinal stem cells (ISCs) (red), *Drosophila* male somatic stem cells (CySCs), and early somatic cyst cells (SCCs) (blue) as well as mouse hematopoietic stem cells (green). 716 genes were found in all four conditions, 354 genes are specific in the testis soma dataset, 1,650 in *Drosophila* ISCs, 435 in *Drosophila* NBs, and 4,388 in mouse hematopoietic stem cells.

(B) Heatmap displaying presence of genes belonging to higher-order categories in different stem cell systems. The color range corresponds to the centered and scaled (per column) fraction of genes annotated to the category that also appear in the sample; red represents high values, and blue represents low fractions of genes in the category that are also present in the sample. Rows and columns are hierarchically clustered using Euclidean distance with complete linkage.

(C–T) L3 wild-type (C–E), *c587 > hyx*^{RNAi} (F–H), *c587 > CtBP*^{RNAi} (I–K), *c587 > fs(1)h*^{RNAi} (L–N), *c587 > Rbf*^{RNAi} (O–Q), and *c587 > DOR*^{RNAi} (R–T) testes were stained for Vas (red) to label the germline, TJ (magenta) to label CySCs and early SCCs, actin (green) to mark cyst cells, and DAPI (blue) for DNA. Insets in (L) and (O) show overproliferation of early germ cells indicated by bright DAPI staining. Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S4.

soma-specific interference with Hyx, a member of the Paf1 protein complex (Mosimann et al., 2009), and CtBP resulted in a reduction of TJ positive somatic cells (Figures 4C, 4E, 4F, 4H, 4I, and 4K). These somatic defects were accompanied with a reduction or absence of spermatogonial cysts and premature germline differentiation (Figures 4D, 4G, and 4J). Knockdown of DOR, a transcriptional co-activator of Ecdysone receptor signaling (Francis et al., 2010), the bromodomain protein Fs(1)h (Florence and Faller, 2008), and Rbf increased the number of TJ-positive somatic cells (Figures 4L, 4N, 4O, 4Q, 4R, and 4T) and induced overproliferation of early germ cells in *fs(1)h*^{RNAi} and *Rbf*^{RNAi} testes (Figures 4M and 4P) (Dominado et al., 2016) and germline differentiation defects in *DOR*^{RNAi} testes (Figure 4S) (Table S5). We next asked whether the commonly expressed TFs function also in other stem cell systems. Thus, we reduced their activity in *Drosophila* ISCs using the *esg*-GAL4 driver (Micchelli and Perrimon, 2006). We found again a high portion (50%) of the testable TF-encoding genes to affect stem cell proliferation in the *Drosophila* intestine (Table S5), as stem cell numbers were either decreased (Figures S4A–S4M) or increased (Figures S4N and S4P–S4S) in knockdown conditions. Interestingly, while seven TFs had a similar effect in both stem cell systems, eight of them affected stem cell behavior in a different manner in the *Drosophila* testis and intestine (Table S5).

In sum, combining expression data from diverse stem cell systems using our online tool resulted in the definition of stem cell-specific and common signatures as well as a unique core gene set. This core gene set contains a high proportion of functionally relevant stem cell regulators. In total, 67% of the 18 TF-encoding genes expressed in the four stem cell types produced RNAi induced phenotypes in the *Drosophila* testis and intestine, which was 5- to 10-fold higher compared to phenotypes found in stem cells by genome-wide RNAi screening (Neumüller et al., 2011; Zeng et al., 2015). Importantly, the fact that many of the commonly expressed TF encoding genes caused divergent phenotypes in the different stem cell types highlighted that these genes are not simply required for general cell survival but rather fulfill specific regulatory roles across systems.

Looking beyond TFs, we identified among the 716 commonly expressed transcripts components of protein complexes thought to execute general cellular functions, including nucleoporins (Nups), building blocks of the nuclear pore complex (NPC), and subunits of the vacuolar (H^+)-ATPase (V-ATPase) proton pump. Recent data show that these protein complexes are more dynamic than previously thought and that individual subunits can have diverse and cell-type-specific functions (Toda et al., 2017; Tognon et al., 2016). Thus, we assumed these proteins to control stem cell behavior and decided to study them in more detail.

A Regulatory Network of Transport: NPC

NPCs are nuclear-envelope-embedded protein assemblies that are composed of more than 30 different Nups, creating a selective transport channel between the nucleus and the cytoplasm (D'Angelo and Hetzer, 2008). In addition, Nups control chromatin organization, gene expression and regulation, cell cycle progression, signaling, and differentiation (Pascual-Garcia and Capelson, 2014; Van de Vosse et al., 2013) and have emerged as potential regulators of stem cell activity (Chen et al., 2013a; Toda et al., 2017).

To elucidate the cell-type-specific function of Nups in the *Drosophila* testis, we interfered with the function of two NPC scaffold proteins (Nup44A and Nup93-1), one Nup located in the cytoplasmic ring (Nup358), and Nup205, which we found to be specifically expressed in the somatic lineage (Figure 5C), in the testis soma. In all cases, we observed an expansion of cells expressing $LamDm_0$ (Figures 5A, 5B, and S5A–S5D), which strongly labels GSCs, gonialblasts, and spermatogonia (Chen et al., 2013a). This showed that early germ cells overproliferated at the expense of differentiation, as LamC-positive spermatocytes were significantly reduced (Figures S5I–S5K, S5M, and S5N). As a consequence, 2, 4, and 8 spermatogonia were lost, and the testes were filled with small $LamDm_0$-labeled germ cells (Figures 5A, 5B, and S5A–S5D). Germline overproliferation is observed when SCCs die (Lim and Fuller, 2012) or are unable to properly encapsulate the germline (Dominado et al., 2016; Li et al., 2003). By analyzing the distribution of βPS-integrin, a marker for cyst cell extensions that encapsulate the germ cells (Papagiannouli et al., 2014), we found that in the absence of Nup205 SCCs were unable to properly wrap the overproliferating germ cells (Figures 5G and 5H). In addition, TJ-positive cells were lost (Figures 5D–5F), and analysis with the cell death marker caspase-3 (Casp-3) revealed induction of apoptosis in *c587 > nup205^{RNAi}* testes (Figures 5J and 5K). Viability of the somatic cell population was restored when apoptosis was prevented by co-expression of the anti-apoptotic baculovirus protein p35 (Hay et al., 1994) (Figures 5I, 5L, S5E, and S5F). Suppression of apoptosis seemed to partially rescue the developmental defects, since *c587 > p35;nup205^{RNAi}* testes contained normal spermatocytes and spermatogonial cysts reappeared (Figures 5A and 5L). These results indicated that the germline lineage was at least partially able to enter the transit-amplifying (TA) program even in the absence of Nup205 when the somatic cell population was kept alive. Importantly, the UAS-*p35*-mediated rescue was not due to a titration of the *nup205^{RNAi}*-induced phenotype by the presence of a second UAS construct (*UAS-p35* and *UAS-nup205^{RNAi}*), as testes co-expressing the UAS-*nup205^{RNAi}* and UAS-*mCD8-GFP* transgenes were similar to *c587 > nup205^{RNAi}* testes with respect to size (Figures 5A, 5B, S5G, S5H, S5L, and S5O) and the inability of somatic cells to properly enclose the germline (Figures 5G, 5H, S5G, S5H, and S5L). Interestingly, knockdown of Nup44A, one of the Nups expressed in all four stem cell systems, in the adult intestine resulted in a loss of ISCs and their progenies (Figures S4N and S4O), indicating that Nups function is highly specific in diverse stem cell types.

Taken together, these results showed that Nups have highly specific functions in the *Drosophila* testis, as they are required for the fitness and survival of SCCs, enabling them to properly encapsulate the neighboring germ cells, thereby restricting proliferation of spermatogonia and promoting their differentiation into spermatocytes.

A Regulatory Network of Signal Processing and Signaling

V-ATPases are hetero-multimeric complexes composed of a cytoplasmic domain, V_1, required for ATP hydrolysis and a transmembrane domain, V_0, critical for proton translocation (Stevens and Forgac, 1997). These proton pumps are generally required for acidification of subcellular compartments, membrane trafficking, pH homeostasis, and protein degradation (Forgac, 2007). More recently, different subunits have been shown to control the activity of various intracellular signaling pathways in different cell contexts (Gross et al., 2012; Zoncu et al., 2011; Petzoldt et al., 2013). The communication between the niche and stem cells is dependent on the interplay of various signaling pathways. Thus, we functionally analyzed four of the 16 genes encoding V-ATPase subunits expressed in the testis soma: Vha16-1 and Vha44, two subunits active in all four stem cell systems and, according to our prediction, active in CySCs and early SCCs (Table S1); and Vha13 and VhaAC45, two subunits found among the gene class 7 (Figure 3B). First, we showed exemplarily for Vha13, a component uniformly expressed throughout the somatic lineage (Figures S6A and S6B), and Vha45AC that they controlled endolysosomal acidification in the *Drosophila* testis, as Lysotracker-488, a pH-sensitive green fluorescent dye, was strongly reduced in *Vha13*- and *Vha45AC*-depleted testes (Figures S6D and S6F) in comparison to wild-type testes (Figures S6C and S6E). Soma-specific interference with *Vha16-1*, *Vha13*, and *VhaAC45* impaired soma differentiation, as the CySC and early SCC marker TJ was expanded (Figures S7F–S7K). This resulted in cells co-expressing TJ and the late SCC marker Eya up to the

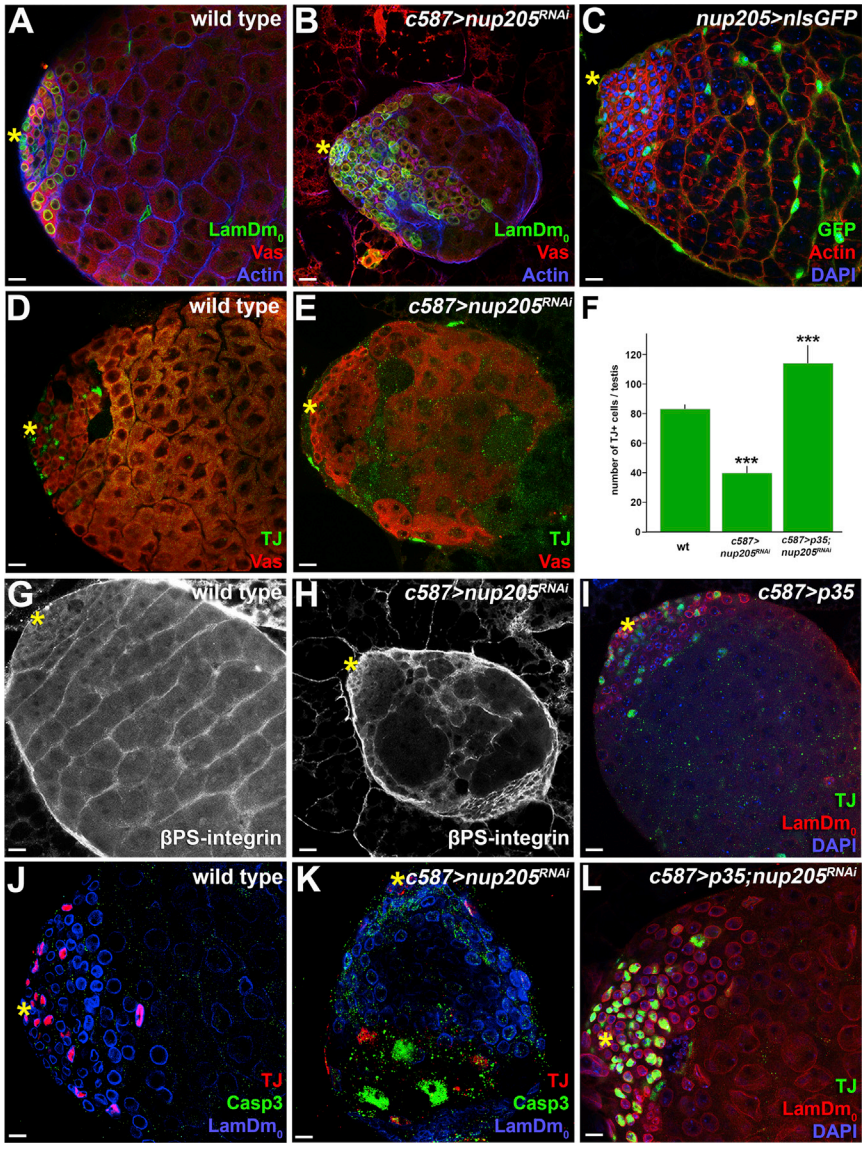

Figure 5. Nucleoporins Have a Critical Function within the Cyst Cell Population and Cell-Non-autonomously Control the Germline of the *Drosophila* Testis

(A and B) L3 wild-type (A) and *c587 > nup205*^{RNAi} (B) testes were stained for Vas (red) to mark the germline, actin (blue) to label cyst cells and germline fusomes, and $LamDm_0$ (green) to indicate early germ cells (GSCs, gonialblasts, and spermatogonia).

(C) *nup205>nlsGFP* L3 testis were stained for GFP (green) and actin (red) to label somatic cyst cells and DAPI (blue) for DNA.

(D and E) L3 wild-type (D) and *c587 > nup205*^{RNAi} (E) testes were stained for Vas (red) to label the germline and TJ to label CySCs and early SCCs.

(F) Quantification of TJ-positive cells in wild-type, *c587 > nup205*^{RNAi} and *c587 > p35;nup205*^{RNAi} testes (***$p < 0.001$).

(G and H) L3 wild-type (G) and *c587 > nup205*^{RNAi} (H) testes were stained for βPS-integrin (white) to indicate cyst cells.

(I) L3 *c587>p35* control testis stained for TJ (green) to label CySCs and early SCCs, $LamDm_0$ (red) to indicate early germ cells, and DAPI (blue) for DNA.

(J and K) L3 wild-type (J) and *c587 > nup205*^{RNAi} (K) testes stained for TJ (red) to label CySCs and early SCCs, $LamDm_0$ (blue) to indicate early germ cells, and caspase-3 (Casp3) (green) to mark apoptotic cells.

(L) *c587 > p35;nup205*^{RNAi} testis stained for TJ (green) to label CySCs and early SCCs, $LamDm_0$ (red) to indicate early germ cells, and DAPI (blue) for DNA. Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S5.

posterior end of the testes (Figures S7I–S7L). This was never observed in wild-type testes, which co-expressed TJ and Eya only close to the hub (Figure S7I). These phenotypically similar defects in somatic cells had different outputs in the germline. In *c587 > Vha16-1*^{RNAi} testes, we identified a reduced number of GSCs as well as clusters of proliferating and unorganized spermatogonia (Figures 6B, 6C, 6E, 6L, and 6N), which were unable to properly differentiate. Germline differentiation defects were confirmed using germ cell-specific, spectrin-rich organelles. In wild-type testes, these organelles were spherical when associated with GSCs and gonialblasts (spectrosomes) and highly branched when germ cells differentiated (fusomes) (Deng and Lin, 1997) (Figure S7A). In *c587 > Vha16-1*^{RNAi} testes, highly branched fusomes were absent and only spherical spectrosomes were found, demonstrating that germline differentiation was aberrant (Figures S7A and S7E). In contrast, spermatogonia in *c587 > Vha13*^{RNAi} and *c587 > VhaAC45*^{RNAi} testes were unable to exit the mitotic cycle and overproliferated, resulting in cysts containing more than 16 spermatogonial cells (Figures 6B, 6C, 6E, 6F, 6H, 6I, and 6K). Concomitantly with their inability to exit mitosis, the germline in *c587 > Vha13*^{RNAi} and *c587 > VhaAC45*^{RNAi} testes displayed differentiation defects, indicated by the presence of spherical or dumbbell shaped spectrosomes throughout the testis (Figures S7A–S7C). The observed phenotype was not due to a loss of interaction between developing germ cells and SCCs, as *Vha13*, *VhaAC45*-depleted, and wild-type SCCs labeled by F-actin enveloped the germ cells equally well (Figures 6D, 6G, and 6J), which was also the case for Vha16-1 (Figure 6M).

V-ATPases has been shown to control various signaling pathways (Gleixner et al., 2014; Petzoldt et al., 2013). Importantly, we found the TOR signaling pathway to be enriched in our datasets (Figure 3C), and network analysis revealed an interaction of V-ATPase subunits with the TOR (Figure 6A). TOR signaling was implicated in the regulation of various mammalian and *Drosophila* stem cell systems (Murakami et al., 2004; Sun et al., 2010) and has recently been shown to control CySC differentiation in the testis (Amoyel et al., 2016b). Similar to the effects

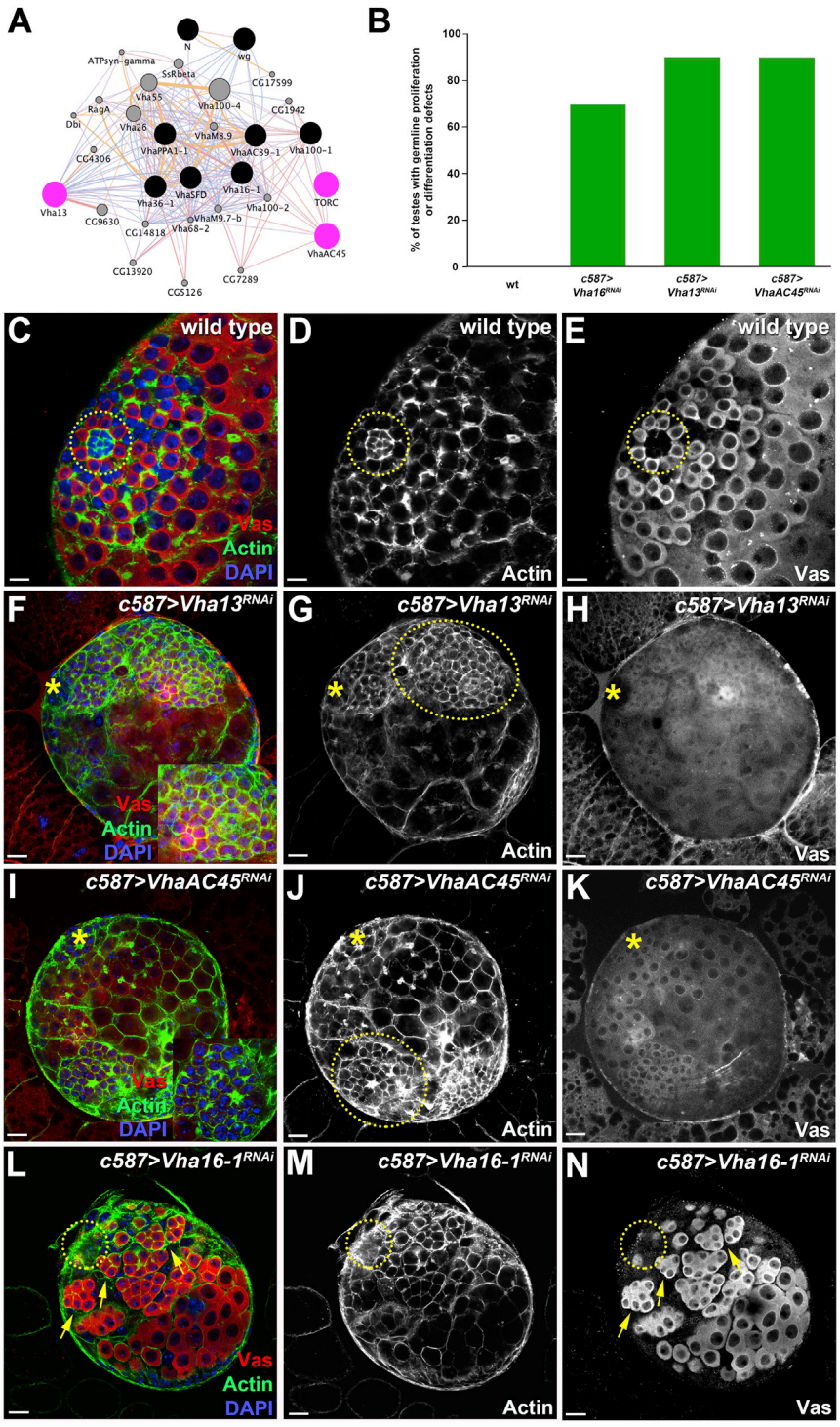

Figure 6. V-ATPases Are Required for Proper Development of the Germline and Somatic Lineage in the *Drosophila* Testis

(A) Gene network showing interactions of V-ATPase subunits and a few signaling components identified in this study; pink indicates genes analyzed in detail.

(B) Quantification of testes displaying proliferation and/or differentiation defects in the germline of the *Drosophila* testis in wild-type, *c587 > Vha16*^{RNAi}, *c587 > Vha13*^{RNAi}, and *c587 > VhaAC45*^{RNAi} animals.

(C–N) L3 wild-type (C–E), *c587 > Vha13*^{RNAi} (F–H), *c587 > VhaAC45*^{RNAi} (I–K), and *c587 > Vha16-1*^{RNAi} (L–N) testes were stained for Vas (red) to label the germline, actin (green) to mark cyst cells, and DAPI (blue) for DNA. The yellow dashed line in (G) and (J) marks overproliferating spermatogonia; in (C) –(E) and (L)–(M), it encircles the hub. The yellow arrows in (L) and (M) highlight unorganized spermatogonia. The insets in (F) and (I) represent high-magnification images. Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S6.

observed in V-ATPase knockdown conditions, and consistent with a previous report (Amoyel et al., 2016b), soma-specific interference with the central pathway player, *Tor*, resulted in an expansion of TJ-positive somatic cells (Figures 7A, 7C–7E, and 7G). This was accompanied with an increase in the number of mitotically dividing spermatogonial cells that were unable to differentiate into mature spermatocytes (Figures 7B and 7F), indicated also by the absence of highly branched fusomes (Figures S7A and S7D). The phenotypic similarities in Tor and V-ATPase subunit knockdown prompted us to test a direct target of Tor phosphorylation, the eIF4-binding (4E-BP) protein (Gingras et al., 1999; Miron et al., 2003), in *Vha13*- and *VhaAC45*-depleted somatic cells using an antibody against p4E-BP. Consistent with a previous report, we detected p4E-BP and thus Tor pathway activation in CySCs and early SCCs of wild-type testes (Figures 7M and 7N) (Amoyel et al., 2016b), which was completely lost in *c587 > VhaAC45*^{RNAi} testes (Figures 7O and 7P). However, *Tor* seemed to function also independently of V-ATPases, since differentiated Eya-positive SCCs were reduced in *Tor* soma-depleted testes (Figures 7H-7L), which was never the case in Vha-13- and VhaAC45-depleted testes (Figures S7I–S7K).

In sum, these data demonstrated that different V-ATPase subunits had highly specific functions in the *Drosophila* testis. Differences in the cell-non-autonomous effects of the Vha16-1 subunit and the Vha13 and VhaAC45 subunits in the germline suggested that these components controlled different signaling pathways required for proper germline differentiation, and we showed that one of them was the TOR pathway.

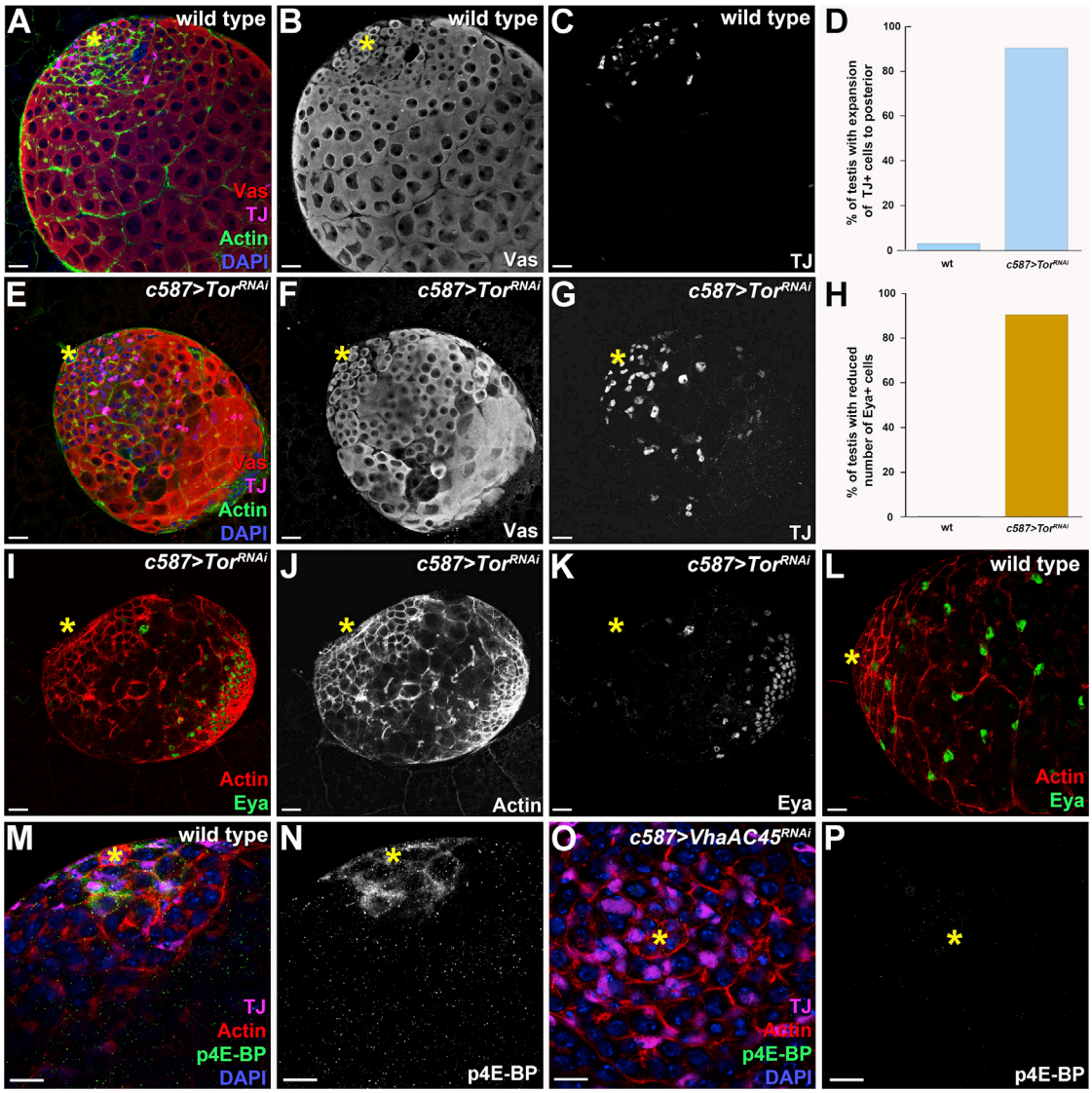

Figure 7. The TOR Pathway Is Required for Proper Development of the Germline and Somatic Lineage and Is Controlled by V-ATPase Subunits

(A–C) L3 wild-type testes were stained for Vas (red) to label the germline, TJ (magenta) to label CySCs and early SCCs, actin (green) to mark cyst cells, and DAPI (blue) for DNA.

(D) Quantification of testes displaying an expansion of TJ-positive cells to the posterior end of the testis in wild-type and *c587 > Tor*^{RNAi} animals.

(E–G) L3 *c587 > Tor*^{RNAi} testes were stained for Vas (red) to label the germline, TJ (magenta) to label CySCs and early SCCs, actin (green) to mark cyst cells, and DAPI (blue) for DNA.

(H) Quantification of testes displaying reduced numbers of Eya positive cells in wild-type and *c587 > Tor*^{RNAi} animals.

(I–L) L3 wild-type (L) and *c587 > Tor*^{RNAi} (I–K) testes were stained for actin (red) to mark cyst cells and Eya (green) to label late SCCs.

(M–P) L3 wild-type (M and N) and *c587 > VhaAC45*^{RNAi} (O and P) testes stained for TJ (magenta) to indicate CySCs and early SCCs, actin (red) to mark cyst cells, p4E-BP (green) to label Tor activity, and DAPI (blue) for DNA. Yellow asterisks mark the location of the hub.

Testes are oriented anterior left. Scale bars, 10 μm. See also Figure S7.

DISCUSSION

Using cell-type-specific transcriptome profiling and *in vivo* TF binding site mapping together with an interactive data analysis tool, we comprehensively identified genes involved in controlling proliferation and differentiation within a stem cell support system. Importantly, many candidates we have functionally tested not only were required within the soma, but also had non cell-autonomous functions in the adjacent (germline) stem cell lineage.

We identified an interconnected network of TFs that play an important role in the maintenance and differentiation of both germline and somatic cell populations, signal processing V-ATPase proton pumps, and nuclear-transport-engaged

Nups as regulators in the *Drosophila* male stem cell system. V-ATPases have been implicated in the regulation of various cellular processes in not only invertebrates but also vertebrates. For example, the V-ATPase subunit V1e1 was previously shown to be essential for the maintenance of NBs in the developing mouse cortex, as loss of this subunit caused a reduction of endogenous Notch signaling and a depletion of NBs by promoting their differentiation into neurons (Lange et al., 2011). Furthermore, two independent studies revealed that V-ATPase subunits and their isoforms are required for proper spermatogenesis in mice, in particular for acrosome acidification and sperm maturation (Imai-Senga et al., 2002; Jaiswal et al., 2014). Thus, it is tempting to speculate that these proton pumps also have important functions in the stem cell pool of the mammalian testis and very likely many other stem cell systems, and we provide some evidence for their crucial role also in ISCs.

This work also uncovered nuclear transport associated proteins, the Nups, as important control hubs in the somatic lineage of the *Drosophila* testis. This is of particular interest, since cell-type-specific functions of Nups have been identified only recently and may represent a critical feature of different stem cell systems. Examples include Nup153, one of the Nups expressed in all four stem cell systems, which interacts with Sox2 neural progenitors and controls their maintenance as well as neuronal differentiation (Toda et al., 2017); Nup358, which plays a role at kinetochores (Roscioli et al., 2012); and Nup98, which regulates the anaphase promoting complex (APC) and mitotic microtubule dynamics to promote spindle assembly (Salsi et al., 2014). Interestingly, it has been shown just recently that Nups play a critical role in regulating the cell fate during early *Drosophila* embryogenesis, thereby contributing to the commitment of pluripotent somatic nuclei into distinct lineages (Hampoelz et al., 2016), and our results suggest that they may play a similar role in controlling the transition of continuously active adult stem cells toward differentiation. The next challenge will be to unravel how variations in the composition of an essential and basic protein complex like the NPC causes differential responses of cells, in particular in stem cells and their progenies.

Our datasets in conjunction with the versatile and easy-to-use analysis tool allowed us to identify a substantial number of stem cell regulators for detailed mechanistic characterization. Importantly, our analyses have shed first light on processes and genes shared between diverse invertebrate and vertebrate stem cell systems and uncovered functionally relevant differences. Owing to its flexibility and the option to include datasets from any species, our online tool represents a valuable resource for the entire stem cell community. It not only provides an open platform for data analysis but also leverages the power of comparative analysis to enable researchers mining genomic datasets from diverse origins in a meaningful and intuitive fashion.

EXPERIMENTAL PROCEDURES

Fly Stocks and Husbandry

See Supplemental Experimental Procedures for a list of fly strains used in this study

Immunofluorescence Staining and Microscopy

See Supplemental Experimental Procedures for detailed list of antibodies used in this study.

DamID

Analysis was performed as previously reported (Papagiannouli et al., 2014). For a detailed protocol, see Supplemental Experimental Procedures.

DamID Analysis and GO term Enrichment Analysis

See Supplemental Experimental Procedures for details.

Interactive Data-Mining Tool

The enrichment analysis method presented in this paper is implemented as a user-friendly Shiny (Beeley, 2016) web application accessible at http://beta-weade.cos.uni-heidelberg.de. The user can select the set of genes to perform the GO enrichment analysis and the respective background independently. Results of the analysis are presented as a plot, an interactive table displaying significantly enriched GO groups, and an interactive heatmap, showing the counts of enriched GO terms within the respective higher-order GO group. It is also possible to get an insight into the individual GO terms that make up a category and into the genes that contributed to the categories or terms. The functionality of the tool exceeds what is described here; a detailed documentation of the tool is deposited under http://beta-weade.cos.uni-heidelberg.de, and an interactive guide is provided in the online application.

DATA AND SOFTWARE AVAILABILITY

The accession number for the genomic data generated in the study is GEO: GSE117833.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.013.

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AUTHOR CONTRIBUTIONS

S.T. conceived, designed, performed, and interpreted experiments on Abd-A, V-ATPases, TOR, and the transcriptional regulators, performed DamID experiments for Zfh-1 and soma and germline PolII TaDa, worked with E.R and O.E. on the interpretation of the bioinformatics data, and wrote parts of the paper; F.P. conceived, designed, performed, and interpreted initial experiments on Abd-A and the nucleoporins, worked together and supervised J.M., N.R., and S.B., established the DamID protocols, and wrote parts of the paper; E.R, O.E., and N.T. designed and implemented the integrative data-mining and analysis tool and assembled with the help of F.P. and S.T. the list of higher-order GO terms; J.M. performed the DamID for Abd-A under the supervision of F.P.; J.Z. conceived, designed, performed, and interpreted experiments on RNAi-based analysis in the *Drosophila* intestine; M.B. conceived

RNAi-based analysis in the *Drosophila* intestine; J.U.L. assisted in designing and implementing the bioinformatics tool; and I.L. conceived the study, assisted in designing and interpreting experiments, designed with E.R, O.E., N.T., and J.U.L. the data-mining and analysis tool, wrote the paper, and obtained funding to support the study (DFG/SFB 873).

DECLARATION OF INTERESTS

The authors declare no competing interests.

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