

A premeiotic function for *boule* in the planarian *Schmidtea mediterranea*

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Mutations in *Deleted in Azoospermia* (*DAZ*), a Y chromosome gene, are an important cause of human male infertility. *DAZ* is found exclusively in primates, limiting functional studies of this gene to its homologs: *boule*, required for meiotic progression of germ cells in invertebrate model systems, and *Daz-like* (*Dazl*), required for early germ cell maintenance in vertebrates. *Dazl* is believed to have acquired its premeiotic role in a vertebrate ancestor following the duplication and functional divergence of the single-copy gene *boule*. However, multiple homologs of *boule* have been identified in some invertebrates, raising the possibility that some of these genes may play other roles, including a premeiotic function. Here we identify two *boule* paralogs in the freshwater planarian *Schmidtea mediterranea*. *Smed-boule1* is necessary for meiotic progression of male germ cells, similar to the known function of *boule* in invertebrates. By contrast, *Smed-boule2* is required for the maintenance of early male germ cells, similar to vertebrate *Dazl*. To examine if *Boule2* may be functionally similar to vertebrate *Dazl*, we identify and functionally characterize planarian homologs of human *DAZL/DAZ*-interacting partners and *DAZ* family mRNA targets. Finally, our phylogenetic analyses indicate that premeiotic functions of planarian *boule2* and vertebrate *Dazl* evolved independently. Our study uncovers a premeiotic role for an invertebrate *boule* homolog and offers a tractable invertebrate model system for studying the premeiotic functions of the *DAZ* protein family.

germ cells | spermatogenesis | Deleted in Azoospermia | *DAZ* | *DAZL*

Human male infertility is often associated with Y chromosome microdeletion (1). In 1976, Tiepolo and Zuffardi proposed the existence of an azoospermia factor (AZF) located on the distal arm of the Y chromosome, which could result in infertility when deleted (2). A strong candidate for AZF is *Deleted in Azoospermia* (*DAZ*), a Y chromosome gene (3, 4). Soon after the discovery of *DAZ*, the mouse and human *DAZ* homolog, *DAZ-like* (*Dazl/DAZL*) (5–7), and the *Drosophila* *DAZ* homolog, *boule* (8), were identified. Phylogenetic analyses showed that *boule* is the ancestral member of the family (9) and is predicted to be present in most metazoans. *Dazl* resulted from duplication of *boule* in an early vertebrate ancestor about 450 million years ago (9). *DAZ*, the newest member of the family, arose from duplication of its autosomal homolog *Dazl* about 30 million years ago (9). The *DAZ* locus is on the Y chromosome and is restricted to humans and Old World monkeys. Thus, in invertebrates, the *DAZ* family is currently represented only by *boule*; nonprimate vertebrates contain both *boule* and *Dazl*; and humans and Old World monkeys possess *boule*, *DAZL*, and *DAZ* (9).

Structurally, *DAZ* family members are characterized by a highly conserved RNA recognition motif (RRM) for binding of target mRNA and a *DAZ* motif for binding of partner proteins. *Boule* and *Dazl* have a single *DAZ* motif, whereas *DAZ* has multiple *DAZ* repeats in tandem (3). Functionally, members of the *DAZ* family are known to play important roles in both male and female germ cell development, although *boule*, *Dazl*, and *DAZ* function at different stages of gametogenesis.

boule appears to function in meiotic or postmeiotic germ cells. Disruption of *boule* in *Drosophila melanogaster* results in male

germ cell meiotic arrest at the G2/M transition, whereas female flies are unaffected (8). In *Caenorhabditis elegans*, loss of function of the *boule* ortholog *dazl* causes sterility by blocking oocytes at the pachytene stage of meiosis I (10). Male *boule* knockout mice are not capable of spermatid maturation, and there is no effect on female gametogenesis (11).

In contrast to *boule*, *Dazl* appears to have an earlier role in germ cell maintenance. *Xenopus* *Xdazl* is present in the germ plasm (12) and in the absence of functional maternal *Xdazl*, primordial germ cells (PGCs) in tadpoles are specified, but fail to differentiate (13). In zebrafish, *zDazl* is expressed in germ plasm of oocytes, activates *tudor domain containing protein 7* (*tdrd7*), and antagonizes miR-430, a microRNA that represses *tdrd7* and *dazl* mRNAs in PGCs (14). In *Dazl*-deficient mice of mixed genetic background, *A*_{aligned} spermatogonia are unable to differentiate (15). In C57BL/6 mice, *Dazl* is first expressed at embryonic day 11.5 (E11.5) (16) and is essential for the survival of both male and female germ cells (17, 18). In male *Dazl* null mice, PGCs are specified and reach the gonad, but by E15.5, show reduced expression of typical germ cell markers and undergo apoptosis (17). Thus, in vertebrates *Dazl* plays a role before meiosis. Finally, Y chromosome deletions spanning the *DAZ* gene are the best-known molecular cause of human male infertility (3, 19), resulting in

Significance

The Deleted in Azoospermia (*DAZ*) family of RNA-binding proteins, consisting of *Boule*, *Daz-like* (*Dazl*), and *DAZ*, plays important roles in gametogenesis. Here we demonstrate that *boule2* in the freshwater planarian *Schmidtea mediterranea* is necessary for the maintenance of early male germ cells, similar to the function of its vertebrate ortholog, *Dazl*. Our results are significant in that a premeiotic role for an invertebrate *boule* homolog has not been described to date. Furthermore, we functionally characterize planarian homologs of human *DAZL/DAZ*-associated proteins and mRNA targets. Our study alters the current understanding of *DAZ* family evolution and establishes *S. mediterranea* as a tractable model organism for the study of premeiotic functions of the *DAZ* family, and its binding partners and targets.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. KU519616 (*boule1*), KU519617 (*boule2*), KU852687 (CDC25-1), KU852688 (CDC25-2), KU852689 (CDC25-3), KU852669 (DAZAP1), KU852670 (DAZAP2), KU852671 (DZIP), KU852676 (GRSF1-1), KU852677 (GRSF1-2), KU852680 (PAM), KU852681 (Pumilio), KU852682 (Ringo/SPY), KU852686 (SDAD1), KU852672 (TPX1), KU852673 (TRF2-1), KU852674 (TRF2-2), KU852675 (TRF2-3), KU852683 (TSSK), KU852684 (Vasa1), and KU852685 (Vasa2)].

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a range of male germ-line phenotypes from complete absence of germ cells to sperm maturation defects (3).

Many years of work have led to a consensus with regard to when the functional divergence between meiotic *boule* and premeiotic *Daiz/DAZ* occurred (9, 20–22). Based on the roles members of this family play across different phyla, it has long been assumed that vertebrate *DAZ* homologs acquired a premeiotic function following duplication of *boule* in a vertebrate ancestor. This hypothesis was proposed based on phylogenetic analysis of both gene families as well as the finding that more exon–intron splicing sites are shared between human *BOULE* and *DAZL* than between human *BOULE* and *Drosophila* *boule*. In addition, human *BOULE* and *DAZL* have an identical number of exons, suggesting a close relationship between vertebrate *DAZ* homologs (9). Based on studies performed in *C. elegans* and *D. melanogaster*, it was also thought that invertebrates only had a single representative of the *DAZ* family; however, it was recently shown that the flatworm *Macrostomum lignano* has three paralogs of *boule* (*macbol1*, *macbol2*, and

macbol3) (21). RNA interference (RNAi) against *macbol2* yielded no detectable phenotypes, *macbol1* RNAi resulted in accumulation of primary spermatocytes and degeneration of more differentiated germ cells of testes, and *macbol3* was required for oocyte maturation and female fertility. This study raised several questions: Do other invertebrates have multiple *DAZ* family members? If so, do any of these invertebrate paralogs play a premeiotic role in germ cell development? Is the premeiotic function of this protein family indeed derived, as currently hypothesized? We addressed these questions using the planarian *Schmidtea mediterranea*, a freshwater flatworm that has emerged as an important model for studying regeneration and germ cell biology (23–29).

Results and Discussion

***S. mediterranea* Has Two Homologs of *boule* That Perform Different Functions in Spermatogenesis.** We identified two planarian *boule* homologs, *boule1* and *boule2*, from the *S. mediterranea* genome database (30) based on the presence of highly conserved RRM

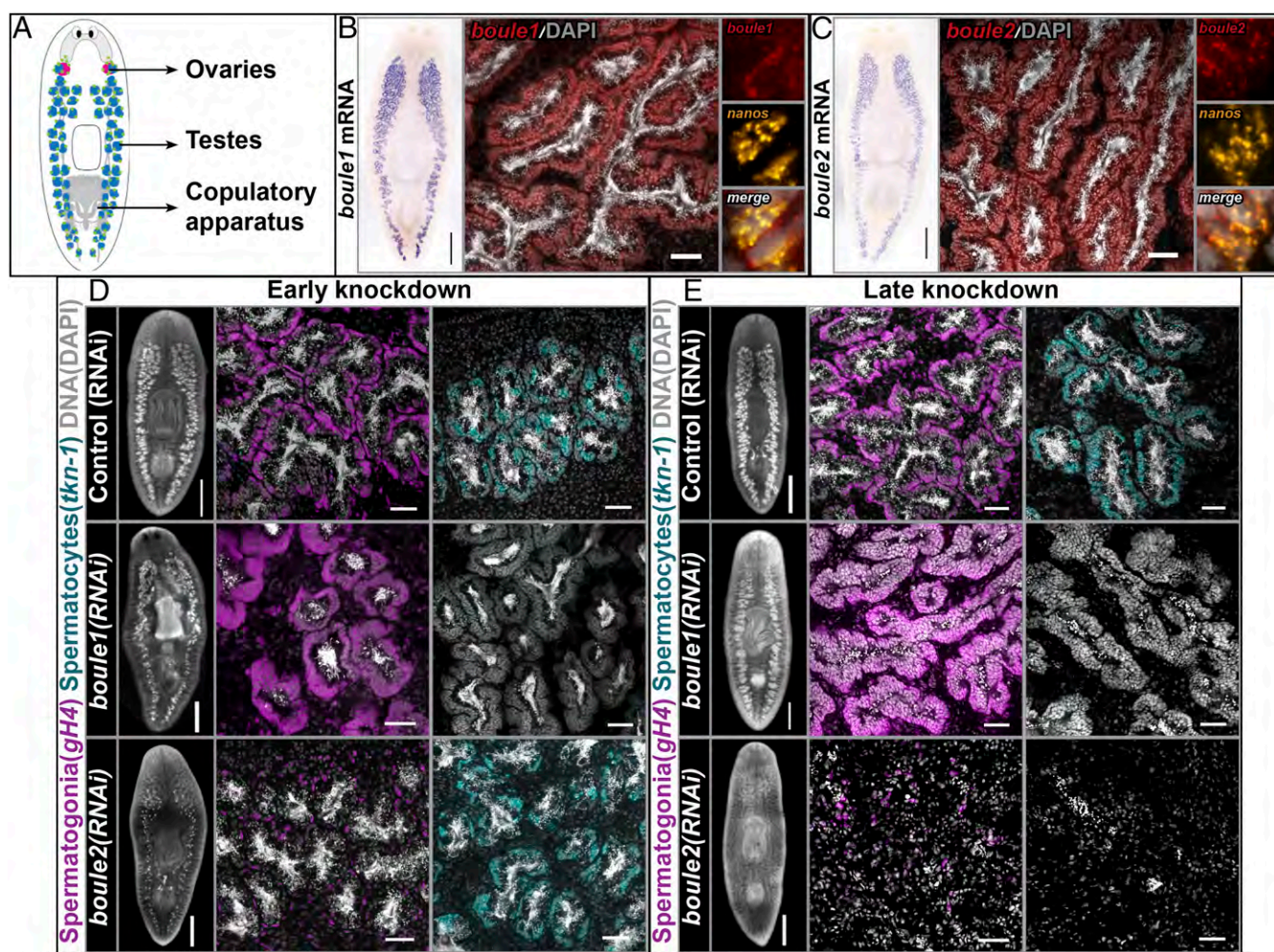


Fig. 1. Planarian *boule1* and *boule2* perform different functions in spermatogenesis. (A) Illustration of sexual planarian depicting the positions of reproductive structures. Ovaries are in red, testes are in blue, and germ-line stem cells are in green. (B and C) Colorimetric ISH showing *boule1* and *boule2* mRNA expression in the testes. (Scale bars, 1 mm.) FISH detects *boule1* and *boule2* expression in spermatogonial stem cells (SSCs), spermatogonia, and spermatocytes. (Scale bars, 50 μ m.) Coexpression of *boule* transcripts with *nanos*⁺ SSCs is shown. (D) Animals fixed following two feedings of dsRNA spaced 4–5 d apart. Control (RNAi), *boule1*(RNAi), and *boule2*(RNAi) animals labeled with germinal histone H4 (gH4) in magenta to detect mitotic spermatogonia and *tektin-1* (*tkn-1*) in cyan to mark meiotic spermatocytes. *boule1*(RNAi) animals show absence of meiotic labeling, but expansion of spermatogonia. The spermatogonial layer is reduced in *boule2*(RNAi) animals, whereas the spermatocyte population is comparable to controls. (E) Animals fixed following four feedings of dsRNA spaced 4–5 d apart. *boule1*(RNAi) testes contain clusters of SSCs and spermatogonia; meiotic and postmeiotic male germ cells are absent. *boule2*(RNAi) animals show a loss of all male germ cells. The remaining gH4 label coincides with neoblasts (somatic stem cells). Left in D and E show whole-mount images. (Scale bars, 1 mm.) Middle and Right in D and E show high magnification view of testis lobes. (Scale bars, 50 μ m.)

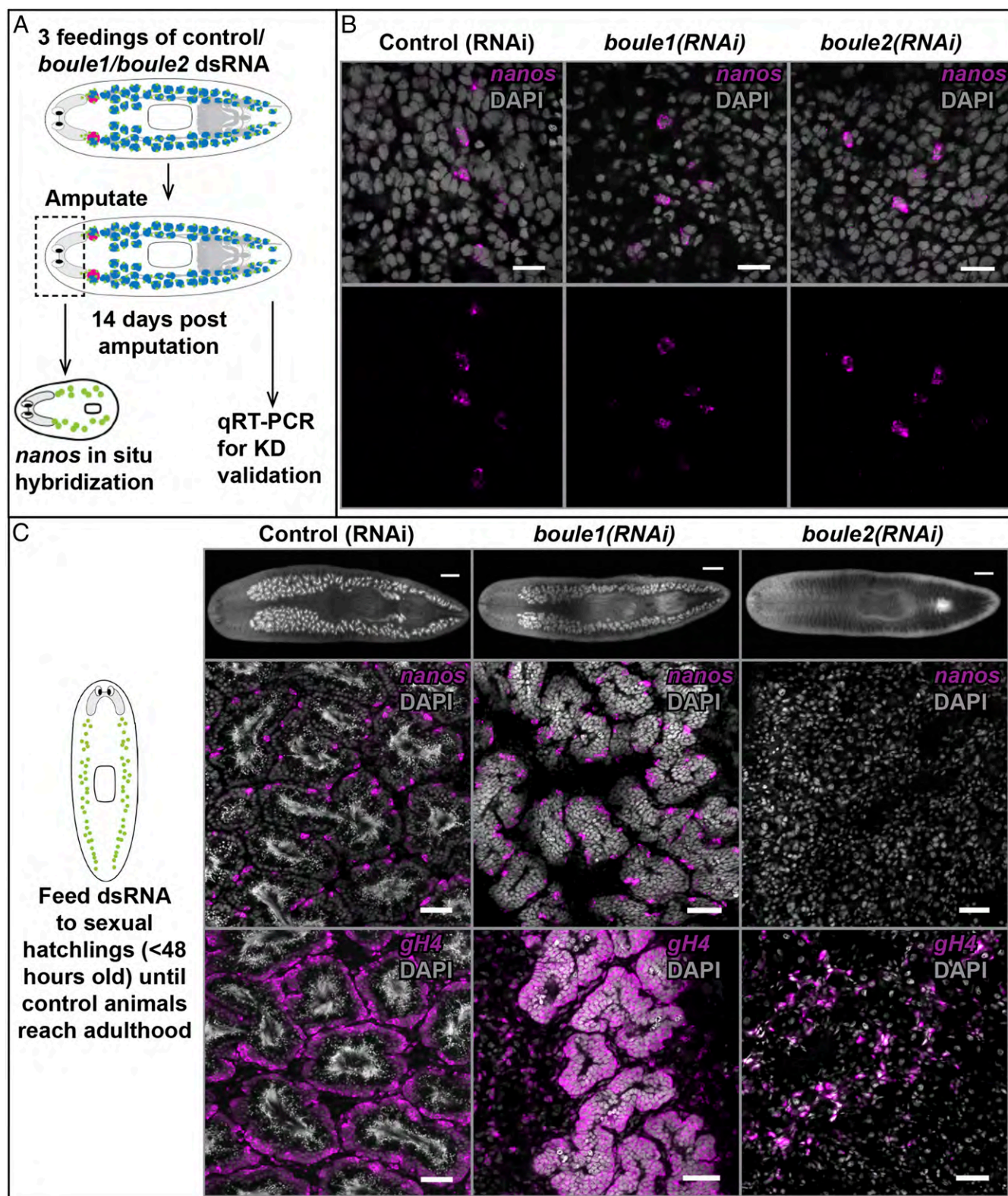


Fig. 2. *boule2* is required for maintenance of early male germ cells but not required for respecification of SSCs. (A) Experimental scheme for testing the requirement of a gene for de novo respecification of SSCs. Animals are fed control/*boule1/boule2* dsRNA three times and amputated anterior to the ovaries. Head fragments, lacking reproductive structures, are allowed to regenerate. Tail fragments are also maintained for knockdown validation. At 14 d following amputation, head fragments are fixed for *nanos* FISH and RNA is extracted from the tail fragment to ensure that test mRNA levels are reduced. *nanos* labels planarian SSCs. (B) Control (RNAi), *boule1*(RNAi), and *boule2*(RNAi) animals all show respecification of *nanos*⁺ SSCs. (Scale bars, 100 μ m.) (C) Sexual hatchlings (<48 h old) are fed liver containing dsRNA until control animals are sexually mature (~10–12 feedings over ~2 mo). SSCs in control (RNAi) animals differentiate and form mature testes. *boule1*(RNAi) animals have testis lobes with only SSCs (*nanos*⁺) and spermatogonia (*gH4*⁺). *boule2*(RNAi) animals lack male germ cells; remnant *gH4* signal is due to neoblasts. (Scale bars, 50 μ m.)

and DAZ motifs characteristic of DAZ family members. To determine the spatial expression of these genes, we performed colorimetric in situ hybridization (ISH) on sexual adults (illustration in Fig. 1A). Both *boule1* and *boule2* were expressed in male and female gonads (Fig. 1B and C and *SI Appendix*, Fig. S1). To determine which specific cells in the testes expressed these transcripts, we performed fluorescence in situ hybridization (FISH) followed by confocal imaging. *boule1* and *boule2* mRNAs were detected in spermatogonial stem cells (SSCs) (Fig. 1B and C and *SI Appendix*, Fig. S2), spermatogonia (*SI Appendix*, Fig. S2), and spermatocytes, to a lesser extent in spermatids, and were absent from mature sperm.

SSCs of *S. mediterranea* give rise to spermatogonia, which undergo three rounds of mitosis with incomplete cytokinesis to generate cysts containing eight primary spermatocytes. These meiotic spermatocytes generate 32 spermatids that mature into sperm (*SI Appendix*, Fig. S3A) (25). We will refer to SSCs and spermatogonia as early male germ cells to distinguish them from the more differentiated meiotic and postmeiotic germ cells. We have previously identified markers for various stages of planarian spermatogenesis (*SI Appendix*, Fig. S3A) (23, 24, 26, 28). RNA ISH using these markers enables us to assess which male

germ cell population is affected following gene knockdown experiments.

To determine the roles of *boule1* and *boule2* in testes, we knocked them down by RNAi and observed effects during homeostasis (in uninjured animals). In early stages of *boule1*(RNAi) (two feedings, 4–5 d apart), *tektin-1*⁺ (*tkn-1*⁺) primary spermatocytes (28) were absent ($n = 6/6$, Fig. 1D). This spermatocyte loss was accompanied by a concomitant increase in the *germinal histone H4*⁺ (*gH4*⁺) mitotic spermatogonial layer (23, 24) ($n = 6/6$, Fig. 1D). At this RNAi timepoint, *boule1*(RNAi) animals showed no discernible changes in the *nanos*⁺ SSC population (*SI Appendix*, Fig. S3B). The *protein kinase A*⁺ (*pka*⁺) spermatid population is slightly reduced in *boule1*(RNAi) animals, possibly as a secondary effect of spermatocyte loss (*SI Appendix*, Fig. S3B). In late stages of *boule1*(RNAi) (four feedings, 4–5 d apart), the testes contained expanded clusters of spermatogonia, with numbers of SSCs comparable to control animals; more mature, meiotic, and postmeiotic male germ cells were absent (Fig. 1E and *SI Appendix*, Fig. S3C).

By contrast, in early *boule2* knockdown animals, there was a reduction in *gH4*⁺ spermatogonia ($n = 5/5$, Fig. 1D), but *tkn-1*⁺ meiotic spermatocytes remained comparable to control animals

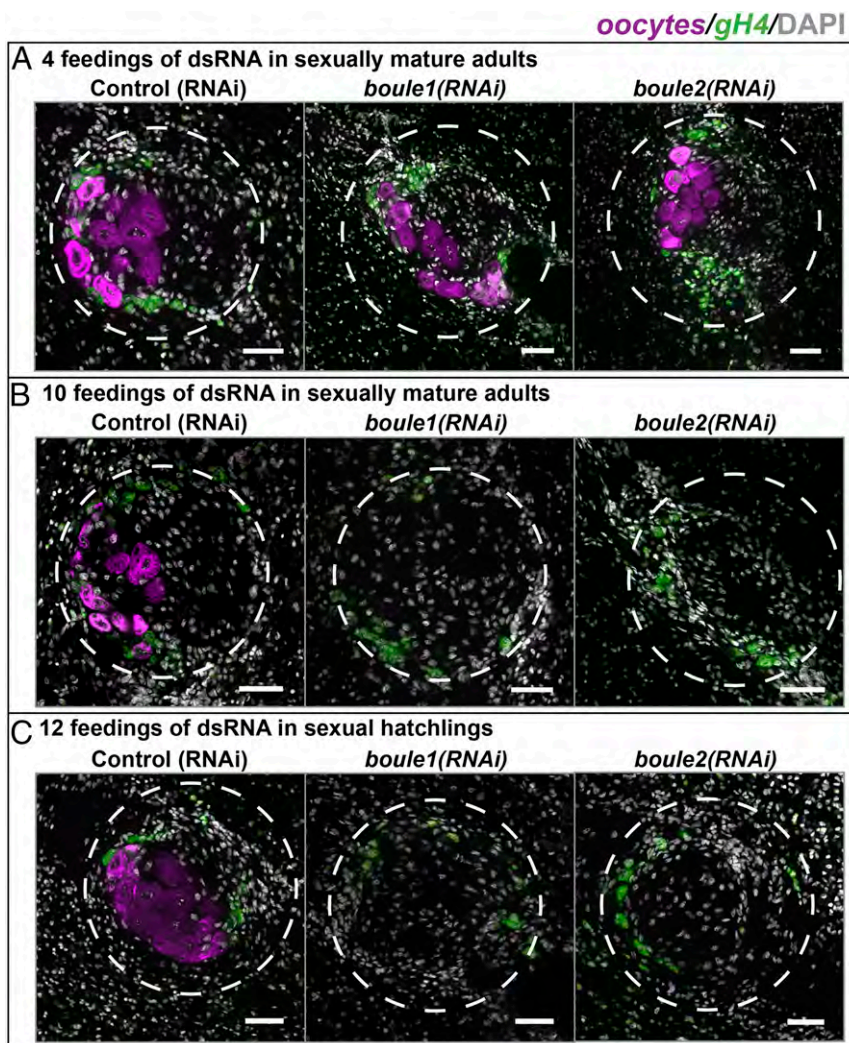


Fig. 3. Planarian *boule* genes play a role in the ovaries. (A) Following 4 feedings of dsRNA, *boule1*(RNAi) and *boule2*(RNAi) female gonads appear similar to controls. Oocytes are marked using *Contig2621* (magenta) (26), and *gH4* (green) labels early female germ cells. (B) Following 10 feedings of dsRNA, *boule1*(RNAi) and *boule2*(RNAi) ovaries have early female germ cells, but lack oocytes. (C) A similar absence of oocytes was seen when sexual hatchlings were fed dsRNA over a period of 2 mo. Dashed circles outline the ovaries. (Scale bars, 50 μm.)

($n = 5/6$, Fig. 1D). Half of *boule2(RNAi)* animals ($n = 3/6$) had no *nanos*⁺ SSCs (SI Appendix, Fig. S3B); *pka*⁺ spermatids appeared unaffected in *boule2(RNAi)* animals at these early stages (SI Appendix, Fig. S3B). We validated the specificity of the gene knock-downs to ensure that RNAi of either *boule1* or *boule2* did not directly affect the other paralog (SI Appendix, Fig. S4). To examine whether early germ cells were being lost at least in part due to apoptosis, we performed TUNEL staining on early *boule2(RNAi)* animals and found that these animals showed an increase in apoptosis compared with control or *boule1(RNAi)* animals (SI Appendix, Fig. S5). In late stages of *boule2(RNAi)*, there was a complete loss of all male germ cells (Fig. 1E and SI Appendix, Fig. S3C).

From our RNAi experiments, we conclude that *boule1* is required for the maintenance and/or formation of meiotic male germ cells. The meiotic role of planarian *boule1* is in agreement with known functions of *boule* orthologs in other systems. However, *boule2* is required for the maintenance of premeiotic male germ cells, SSCs and spermatogonia, remarkably similar to

the function of mouse *Dazl* (7, 15, 17). When *boule2* expression is inhibited, the early germ cells appear to undergo increased apoptosis (SI Appendix, Fig. S5).

***boule2* Is Required for the Maintenance, but Not Specification, of Early Male Germ Cells.** In addition to their remarkable ability to regenerate all body parts and organ systems, planarians are capable of respecifying germ cells from amputated tissue fragments devoid of reproductive structures (24, 25, 29). Thus, like mammals, planarians can specify their germ line via inductive signals. Within 2 wk of regeneration, germ cells are respecified, as determined by the expression of *nanos*, the earliest known marker expressed in planarian germ cells (schematic in Fig. 2A) (24, 29). We examined whether *boule1* or *boule2* is required for respecifying germ cells by knocking down the corresponding genes before amputation.

We found that both *boule1* and *boule2* were dispensable for the regeneration of *nanos*⁺ SSCs ($n = 10/10$ for both, Fig. 2B). As an

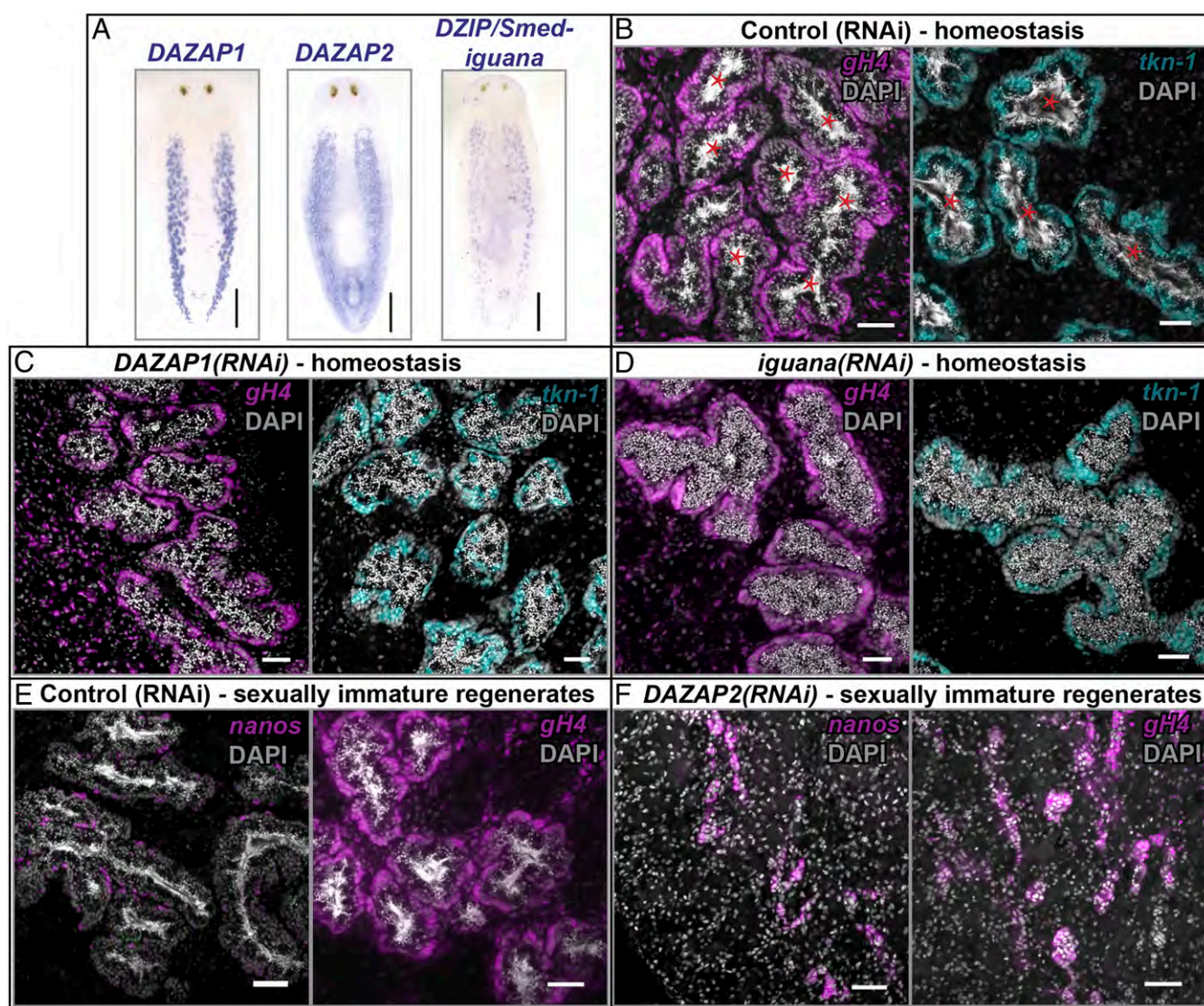


Fig. 4. Homologs of vertebrate DAZ-associated proteins are expressed and function in the testes of *S. mediterranea*. (A) *DAZAP1*, *DAZAP2*, and *DZIP* (*Smed-iguana*) transcripts are detected in the testes by ISH. (Scale bars, 1 mm.) (B) Adults fed control dsRNA in homeostasis show robust spermiogenesis. Thin, threadlike nuclei of mature sperm in the lumen of testis lobes are labeled with DAPI (marked by red asterisk). Both (C) *DAZAP1(RNAi)* and (D) *iguana(RNAi)* animals lack mature sperm, but have spermatogonia and spermatocytes similar to control (RNAi) animals. (E) Sexually immature regenerates fed control dsRNA regenerate their testes, whereas (F) *DAZAP2* dsRNA-fed regenerates have small testes containing only SSCs and spermatogonia. (Scale bars, 50 μ m.)

boule1 and boule2 Are Necessary for Oogenesis. We examined the role of *boule1* and *boule2* in the ovaries by carrying out gene knockdowns for different lengths of time and during different developmental stages. Following 4 dsRNA feedings (spaced 4–5 d apart), ovaries of *boule1(RNAi)* and *boule2(RNAi)* animals appeared comparable to controls ($n = 6/6$ for all samples, Fig. 3A). However, following prolonged gene knockdown (10 feedings over a period of ~2 mo), both *boule1(RNAi)* ($n = 4/4$) and *boule2(RNAi)* ($n = 6/6$) animals lacked oocytes, whereas early *gH4⁺* female germ cells were still present (Fig. 3B). Similarly, when sexual hatchlings were fed *boule1* and *boule2* dsRNA over a period of 2 mo, the animals lacked mature oocytes, but *gH4⁺* female germ cells were present ($n = 4/4$ for all samples, Fig. 3C). The dual role of planarian *boule* genes in both testes and ovaries is especially interesting because, with the exception of *Dazl*, other members of the DAZ family (*boule* orthologs in various systems and *DAZ*) appear restricted in function exclusively to the male or the female germ line.

Homologs of Vertebrate DAZ-Associated Proteins Are Expressed and Function in the Testes of *S. mediterranea*. Yeast two-hybrid screens and other in vitro studies (31–34) have identified several potential

DAZL/DAZ-interacting partners using human DAZ as bait. Homologs of these genes have not been described in *C. elegans* and *D. melanogaster*, which only possess meiotic *boule* (*Metho*). To further investigate the functions of these DAZL/DAZ-interacting partners, we sought to identify planarian homologs of DAZ-binding partners.

Using BLAST similarity search, we identified planarian homologs of putative DAZL/DAZ-interacting partners—*DAZAP1*, *DAZAP2*, and *DZIP* (*Smed-iguana*)—and found that these genes were expressed in the testes (Fig. 4A). To determine the role of these genes in spermatogenesis, we performed RNAi during homeostasis (in sexually mature adults), during development (in hatchlings), as well as in sexually immature regenerates (animals fed dsRNA three times, amputated prepharyngeally to induce regression of testes (29), and refed dsRNA during regeneration). *DAZAP1(RNAi)* animals lacked elongated spermatids and mature sperm, whereas other male germ cells appeared intact in all three experimental conditions ($n = 6/6$ for all; Fig. 4C and *SI Appendix*, Fig. S8 C, E, and F and Table S1). Therefore, similar to *DAZAP1* knockout mice, which lack mature male gametes (35), *DAZAP1* is required for spermiogenesis.

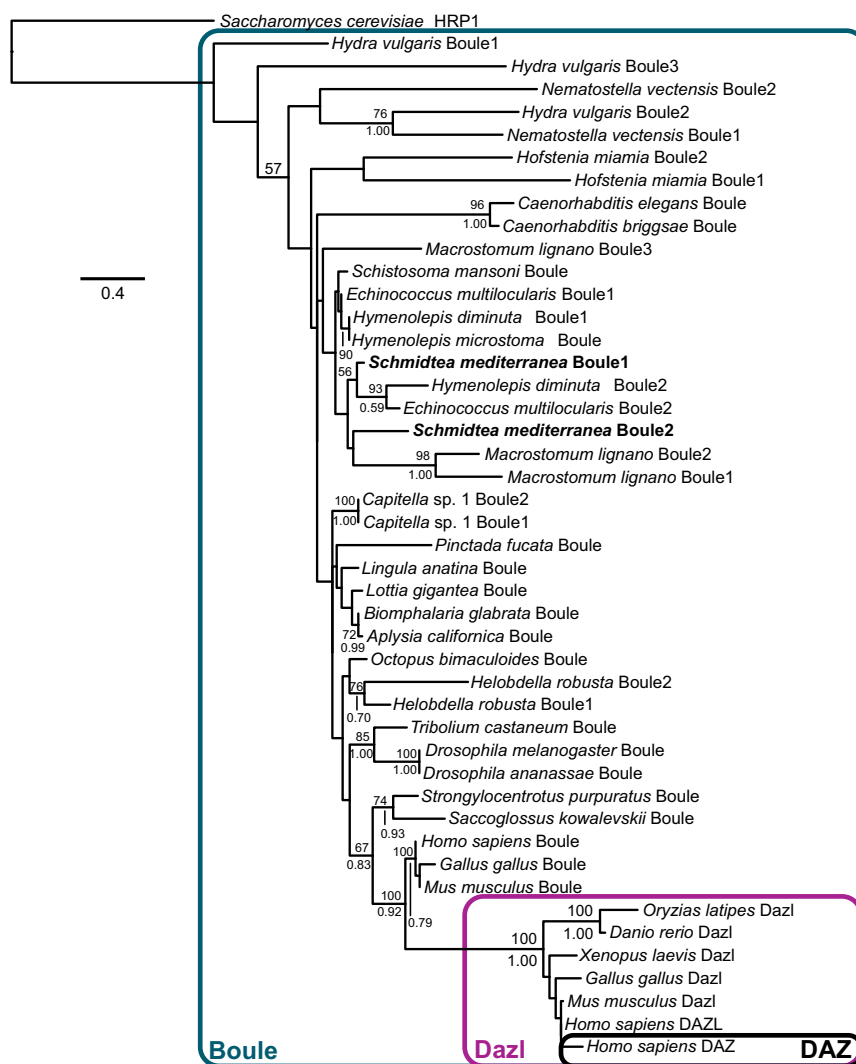


Fig. 6. Phylogenetic analysis reveals independent origins of planarian Boule2 and vertebrate Dazl. Phylogenetic tree topology of DAZ gene family from ML and BI analysis. Numbers above nodes indicate ML bootstrap resampling frequencies (500 replicates). Numbers below nodes indicate Bayesian posterior probability values.

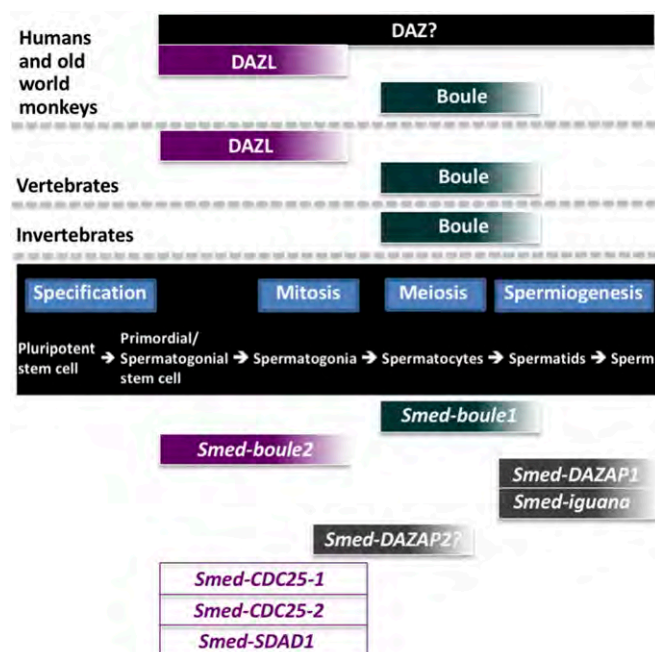


Fig. 7. Summary of DAZ family functions in planarians and other systems. (Center) Different stages of spermatogenesis. (Upper) Known functions of the DAZ family in male germ cell development in other systems. (Lower) Summary of the functions of planarian *boule* genes, their putative-associated proteins, and targets.

DAZAP2 did not have a germ cell RNAi phenotype in sexually mature adults (*SI Appendix*, Fig. S8 A and B) or hatchlings (*SI Appendix*, Table S1). However, regeneration experiments revealed that *DAZAP2(RNAi)* regenerates either lacked testes ($n = 2/6$) or had regressed testis lobes containing only SSCs and spermatogonia ($n = 4/6$) (Fig. 4 E and F). Understanding this regeneration-specific role of *DAZAP2* in male germ cells requires further investigation. *DAZAP1* and *DAZAP2* are not required for respecification of *nanos*⁺ SSCs (*SI Appendix*, Table S1).

The planarian *DZIP* gene, known as *Smed-iguana*, has previously been shown to be required for ciliogenesis in asexual planarians (36). Regenerating *iguana(RNAi)* asexuals are able to produce normal blastemas, but do not form ciliated epidermis (leading to defects in cilia-driven locomotion) or ciliated protonephridia (resulting in bloating and blistering defects due to disrupted osmoregulatory function) (36). *iguana(RNAi)* in the sexual strain led to bloating defects similar to the asexual strain (*SI Appendix*, Table S1). Furthermore, we observed spermiogenesis defects in *iguana(RNAi)* animals (Fig. 4D and *SI Appendix*, Fig. S8D). In addition to defects in the testes, sexually immature *iguana(RNAi)* regenerates underwent lysis during regeneration (*SI Appendix*, Table S1). This lysis phenotype was not reported in asexual planarians; differences in our observations may be explained by differences in dsRNA-treatment regimes.

Planarian *DAZAP1*, *DAZAP2*, and *iguana* play roles in spermatogenesis, but the knockdown of these genes does not phenocopy *boule1(RNAi)* or *boule2(RNAi)*, in that these genes appear to be required for later stages of germ cell maturation. Several possibilities may explain this finding. Because there are multiple DAZ binding partners, knockdown of one factor alone may not be sufficient to recapitulate the *boule1/2(RNAi)* phenotype. *iguana* could have pleiotropic effects as it is also required for regeneration and osmoregulation. Alternatively, *boule1* and *boule2* may play a role in postmeiotic spermatid elongation and maturation (similar to *DAZAP1* and *iguana*), but the rapid loss of meiotic and premeiotic germ cells may not allow us to observe

these possible secondary, less obvious effects. It is also possible that the gonadal function of these putative binding partners is independent of *boule1* or *boule2*. Together, our data support roles for planarian *DAZAP1*, *DAZAP2*, and *iguana* in male germ cell differentiation (*SI Appendix*, Table S1).

Transcripts of other broadly conserved DAZ family interacting partners, such as Pumilio and Poly(A) Binding Protein (PABP) (34, 37, 38), are also enriched in planarian testes. *pumilio(RNAi)* is lethal, consistent with a similar observation in the planarian *Dugesia japonica* (39), and specific germ cell defects were not detected before death (*SI Appendix*, Table S1). Knockdown of planarian *PABPC* has been described previously (26) and is remarkably similar to the *boule1(RNAi)* phenotype in that meiotic and postmeiotic male germ cells are lost with a concomitant accumulation of spermatogonia. The identification of these homologs of vertebrate DAZ-associated proteins in *S. mediterranea* is promising as it allows functional studies of these genes and other putative DAZ-associated proteins in a tractable invertebrate model system.

Knockdown of Putative Planarian DAZ Family Targets Phenocopies *boule2(RNAi)*. Several in vitro studies have identified presumptive mRNA targets for the DAZ protein family, but to what extent these targets overlap between different orthologs (*Boule*, *Dazl*, and *DAZ*) is uncertain (40–44). We identified and cloned a number of planarian homologs of putative DAZ family targets (Fig. 5A and *SI Appendix*, Fig. S9A and Table S2), but we will focus on the putative targets with germ cell RNAi phenotypes.

SDAD1, a homolog of the yeast gene *severe depolymerization of actin*, is a putative target of human *DAZL* and *PUMILIO 2* (44). A function for *SDAD1* in spermatogenesis has not been reported previously. By ISH, we find that *Smed-SDAD1* was detected in the testes as well as soma (Fig. 5A). RNAi experiments showed that *SDAD1* is required for maintenance of SSCs ($n = 3/6$) and spermatogonia ($n = 6/6$), similar to *boule2(RNAi)* (Fig. 5C and *SI Appendix*, Fig. S9C). *SDAD1(RNAi)* animals undergo lysis upon continued knockdown or when amputated (*SI Appendix*, Table S2), indicating a possible somatic function and precluding the possibility of testing if *SDAD1* is necessary for specification of early germ cells.

The *CDC25* homolog *twine* is a known target of *Boule* in *D. melanogaster* (40). Two of the planarian homologs of *CDC25* (a somatic planarian *CDC25* homolog has been described previously (45) and will not be discussed here), designated *CDC25-1* and *CDC25-2*, were expressed in testes (Fig. 5A). Interestingly, following three feedings of *CDC25-1* or *CDC25-2* dsRNA in adults, animals showed defects similar to *boule2(RNAi)*: the spermatogonial layer was reduced, whereas the spermatocyte layer appeared intact ($n = 6/6$ for both knockdowns, Fig. 5D and E). The numbers of SSCs and spermatids were largely unaffected at the initial stages of knockdown (*SI Appendix*, Fig. S9D and E); at later stages, all male germ cells were absent (Fig. 5F and *SI Appendix*, Table S2). We next tested the requirement of *CDC25-1* and *CDC25-2* for specification and maintenance of early germ cells in sexual regenerates. *CDC25-1(RNAi)* animals do not regenerate, and undergo lysis, but there are no male germ cells present in regenerates before lysis (*SI Appendix*, Table S2). *CDC25-2(RNAi)* sexual regenerates phenocopy *boule2(RNAi)* regenerates—these animals respecify their SSCs ($n = 9/9$; *SI Appendix*, Table S2), but cannot maintain early germ cell clusters ($n = 6/6$; *SI Appendix*, Fig. S9F and G). The in vitro prediction that these transcripts are DAZ family targets in other systems, combined with the similarity of RNAi phenotypes between these genes and *boule2*, makes these transcripts strong candidates for putative targets regulated by planarian *Boule2*.

Premeiotic Functions of the DAZ Family Evolved Independently in Planarians and Vertebrates. Vertebrate *Dazl*, which plays a premeiotic role in germ cells, arose either during vertebrate evolution, or was present in a last common bilaterian ancestor and was

subsequently lost in some invertebrates. Based on the presence of a single DAZ family representative, *boule*, in both *C. elegans* and *D. melanogaster*, phylogenetic analyses, and comparison of gene structure and intron/exon counts, it has been proposed that *Dazl* arose through duplication of *boule* in the vertebrate stem lineage (9). Our identification of multiple paralogs of *boule* in an invertebrate model system, combined with the premeiotic germ cell function for one of these paralogs, provides us valuable tools for testing this hypothesis in a phylogenetic context.

We obtained multiple *Boule* sequences from diverse animal phyla (accession nos. in *SI Appendix, Table S3*; alignments in *SI Appendix, Fig. S10A*), placing special focus on invertebrates with multiple annotated *Boule* homologs. We performed both maximum likelihood (ML) (46) and Bayesian inference (BI) (47) analyses and found that *S. mediterranea* *Boule* paralogs were recovered in a clade formed by other platyhelminth *Boule* orthologs (Fig. 6). The short patristic distance between *S. mediterranea* paralogs suggests lineage-specific differentiation of premeiotic and meiotic functions of DAZ family members in flatworms and vertebrates. When we enforced a topological constraint to render a single origin of premeiotic *Boule* function, forcing monophyly of planarian *Boule2* and vertebrate *Dazl*/DAZ clade, the constrained tree was significantly less likely than the optimal ML tree (*Methods*). The phylogenetic distance between *S. mediterranea* *Boule* paralogs and their vertebrate orthologs supports a scenario of independent origins of premeiotic DAZ family members in planarians and vertebrates.

To infer whether the premeiotic planarian *Boule* had diverged from its ancestral sequence (an independent test of neofunctionalization) (48, 49), we examined the ratio of branch lengths (sequence divergences) of premeiotic and meiotic DAZ family members in two planarians and three vertebrates, with branch lengths drawn from the Bayesian postburnin tree set (*SI Appendix, Fig. S10B*). For both *S. mediterranea* and the vertebrates, the distributions of ratios of premeiotic paralog branch lengths to meiotic paralog branch lengths were highly comparable, in contrast to the ratio distribution for the *Boule* proteins of *Macrostomum lignano*. This result is consistent with neofunctionalization of planarian and vertebrate premeiotic *Boule* derivatives.

boule, *Dazl*, and *DAZ* play crucial and conserved roles in gametogenesis across the animal kingdom (Fig. 7). However, there is considerable phenotypic diversity caused by defects in the DAZ family of proteins, and our present study adds another dimension to the understanding of these genes. Our study also raises many interesting questions. For instance, have vertebrate DAZ-associated proteins evolved independently in planarians, or have they been lost in Ecdysozoans such as *C. elegans* and *D. melanogaster*, especially in light of the finding that a DAZAP-like protein has been described in the flatworm *D. japonica* (50)? Another interesting question is why some invertebrates have multiple *boule* homologs and others do not. Functional and phylogenetic studies of *boule* genes in other species with multiple *boule* paralogs will open the field to further address these questions and will help illuminate the entire range of functions of the DAZ protein family.

Methods

Planarian Culture. Sexual planarians were maintained in 0.75× Montjuïc salts at 18 °C (24). Animals were fed organic calf liver and starved for 1 wk before use.

Identification and Cloning of *boule* Homologs, Putative Binding Partners, and Targets. Planarian *boule* homologs were identified by the presence of RRM and DAZ motifs and cloned into pJC53.2 (27). The full-length sequence for *boule2* was obtained from PlanMine v1.0 (51). Planarian homologs of putative binding partners and targets were identified from the *Smed* genome database (30), based on sequence similarity to human counterparts. More specifically, the amino acid sequence of human/vertebrate DAZ-associated proteins and targets was obtained from National Center for Biotechnology Information (NCBI) and tblastn analysis was performed in PlanMine v1.0. The top genes obtained from this search were subjected to a reciprocal blastp against NCBI protein databases to ensure that the planarian gene was indeed a homolog of

the human gene. BLAST analysis comparing human DAZAP1 to FlyBase and WormBase revealed a heterogeneous nuclear ribonucleoprotein, the reciprocal protein blast of which to NCBI protein databases did not yield DAZAP1 as the highest hit. No sequences corresponding to DAZAP2 and *DZlpliguna* were found. Cloning primers are in *SI Appendix, Table S4*.

dsRNA Synthesis and RNAi. cDNAs corresponding to *boule1* and *boule2* cloned in pJC53.2 (27) were used as template to generate dsRNA by in vitro transcription (IVT). The 20-μL IVT reaction contains 2 μL 10× high yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl₂, 20 mM spermidine, 0.1 M DTT), 5 μL 25 mM rNTPs (Promega), 1 μL T7 polymerase, 1 μL thermostable inorganic pyrophosphatase (TIPP; 2,000 units/mL; New England Biolabs), 0.5 μL recombinant ribonuclease inhibitor (RNasin; 2,500 units/mL; Promega), and 0.5–2.5 μg of PCR product. Reactions were incubated at 37 °C overnight, then treated with 1 μL of RQ1 RNase-free DNase (Fisher Scientific) for 20 min at room temperature. Each reaction was brought up to 100 μL, followed by denaturing and annealing at the following temperatures: 95 °C (3 min), 75 °C (3 min), 50 °C (3 min), and room temperature (5 min). dsRNA was precipitated using ammonium acetate (2.5 M final concentration) plus two volumes of 100% ethanol. dsRNA (0.4–1 μg) was mixed with 10 μL of 3:1 liver:Montjuïc salts mix. Control animals were fed dsRNA synthesized from a nonplanarian gene inserted in pJC53.2.

Riboprobe Synthesis. *boule1* and *boule2* cDNA cloned in pJC53.2 (27) were used as templates to generate riboprobes. Each 20-μL reaction contained 2 μL 10× high yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl₂, 20 mM spermidine, 0.1 M DTT), 1 μL 10/6 mM rNTPs (CTP, ATP, and GTP 10 mM final, UTP 6 mM final) (Promega), 0.4 μL of Digoxigenin-12-UTP (Roche), 0.6 μL recombinant ribonuclease inhibitor (RNasin, 2,500 units/mL) (Promega), 2 μL of SP6/T3 RNA polymerase, and 0.5–2.5 μg of PCR product. Riboprobes were synthesized for 4–5 h at 37 °C, treated with 1 μL of RQ1 RNase-free DNase (Fisher Scientific) for 20 min at room temperature, and precipitated with ammonium acetate (2.5 M final concentration) plus two volumes of 100% EtOH.

ISH. ISH was performed as described previously (52). Detailed methods are provided in *SI Appendix, SI Methods*.

TUNEL on Sections. The planarian whole-mount TUNEL protocol was modified for cryosections (53, 54). Detailed methods are provided in *SI Appendix, SI Methods*.

Multiple Sequence Alignment and Phylogenetic Analysis. Peptide sequences of 46 *Boule*, *Dazl*, and *DAZ* RRM (accession nos. in *SI Appendix, Table S3*) were aligned using MUSCLE v. 3.8 (55) with default alignment parameters. HRP1 of *Saccharomyces cerevisiae* was used as an outgroup. The sequence alignment is provided as *SI Appendix, Fig. S10A*. Tree topologies were inferred using ML and BI. ML analysis was done using RAxML, 100 independent searches, 500 bootstraps, using LG+Gamma model of evolution (46). BI analysis was done using MrBayes v. 3.2 (47). Four runs, each with four chains and a default distribution of chain temperatures, were run for 2×10^6 generations, with sampling every 2,000th iteration. A mixed+I+G model (56) was implemented, following model selection with ProtTest v.3 (57). Convergence was independently assessed using average split frequency and with Tracer v. 1.6 (58). As a conservative treatment, 5×10^5 generations (25%) were discarded as burnin.

Likelihood Ratio Tests. The strength of phylogenetic evidence for independent origins of premeiotic DAZ family representatives in vertebrates and the planarian was assessed using Shimodaira–Hasegawa (59) and approximately unbiased (60) tests in RAxML v. 7.7.5 (46). Topological constraint to render a single origin of premeiotic function was enforced and the resulting tree topology was compared to our unconstrained ML tree. Per-site log likelihood values were computed using the -f g command in RAxML v. 7.7.5. The resulting likelihoods were analyzed using CONSEL v. 0.1i (61). using 10,000 bootstrap replicates to conduct the tests of monophyly.

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Supporting Information Appendix for:

A Premeiotic Function for *boule* in the Planarian *Schmidtea mediterranea*

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This SI appendix includes:

Detailed Supplementary Methods

Supplementary Figure Legends

Supplementary Figures S1-S10

Supplementary Tables S1-S4

Detailed Supplementary Methods

In situ hybridization

For colorimetric in situ hybridization (ISH) and fluorescent in situ hybridization (FISH), animals were killed with 10% N-acetyl cysteine (Sigma-Aldrich, St. Louis, MO), for 7.5 minutes, and then fixed in 4% formaldehyde in PBSTx (1X PBS+0.3% Triton X-100) for 20 minutes at room temperature. Animals were dehydrated in 50% followed by 100% Methanol and stored in -20°C until use. Animals were rehydrated in increasing concentrations of PBSTx, and bleached in freshly prepared Formamide bleaching solution (5% non-ionized Formamide, 0.5X SSC, and 1.2% H_2O_2) for 3 hours. After bleaching, animals were treated with Proteinase K solution (100 μl of 10% SDS and 5 μl of 20 mg/ml Proteinase K (Invitrogen) in 9.9 ml of PBSTx) and post-fixed in 4% Formaldehyde. Following washes to remove the fixative, hybridization was carried out at 56°C for 16 hours at a riboprobe concentration of 0.1-0.5 ng/ μl . After post-hybridization washes, samples were blocked in Blocking solution (5% horse serum and 0.5% Roche Western Blocking Reagent in MABT). Samples were incubated in primary antibody (anti-digoxigenin alkaline phosphatase (Roche) 1:1000, or anti-digoxigenin peroxidase (Roche) 1:1000) overnight at 12°C . For FISH, DAPI was added to the primary antibody solution (1:10,000 of 10 mg/ml stock). Samples were washed in MABT (100 mM Maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Colorimetric development was carried out in AP buffer (100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM MgCl_2 , 0.1% Tween-20 brought up to volume with 10% polyvinylalcohol (Sigma)) containing 4.5 $\mu\text{l}/\text{ml}$ NBT and 3.5 $\mu\text{l}/\text{ml}$ BCIP (Roche). For FISH, development was done in freshly made Tyramide solution (Fluor-tyramide (1:250-1:500), 4-IPBA (1:1000), and H_2O_2 (0.003%) in TSA buffer (2 M NaCl, 0.1 M Boric acid, pH 8.5). 4-IPBA is 20 mg/ml of 4-iodophenylboronic acid in

dimethylformamide (DMF) stored at -20°C . Samples were washed 6-8 times (~20 minutes each wash) in TNTx.

TUNEL on sections

After two dsRNA feedings planarians were starved for a week and treated with 10% N-acetyl-L-cysteine for 7.5 minutes and fixed in 4% formaldehyde in PBSTx (0.3% Triton X-100) for 20 minutes at room temperature. Cryosectioning was done to generate 15-20 μm sections. Sections were rehydrated and treated with pre-chilled ethanol:acetic acid (2:1) at -20°C for 5 minutes. The slides were rinsed twice in DI water and equilibrated in equilibration buffer (100 mM Tris-HCl pH 7.5 + 1 mg/ml IgG-free BSA). Slides were covered with TdT solution (0.5 μl NEB TdT (Cat. No. M0252L), 2 μl NEB buffer 4, 2 μl 2.5 mM CoCl_2 , 0.8 μl 1:50 DIG-dUTP in dATP, 14.7 μl water) and incubated at 37°C in a dark humidified chamber for 1 hour. After rinsing 3X with PBSTx, the sections were blocked with 5% Horse Serum (Sigma H1138) in PBSTx for 2 hours. Block was replaced with 1:1000 anti-DIG-POD (Roche 11207733910) diluted in block solution. DAPI (1 $\mu\text{g}/\text{ml}$) was added at this step. Sections were covered with coverslips and incubated for overnight at 4°C . Slides were rinsed in PBSTx and signal was revealed using TAMRA-tyramide. Slides were rinsed in PBSTx and mounted in Vectashield.

Imaging

Colorimetric in situ samples were mounted in 80% glycerol and images were captured with a Leica DFC420 camera mounted on a Leica M205A stereomicroscope (Leica, Wetzlar, Germany). Whole-mount FISH samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged on a Zeiss Stereo Lumar V12 (Carl Zeiss, Germany). For confocal imaging, FISH samples were mounted in Vectashield and images were obtained on a Zeiss LSM 710 confocal microscope (Carl

Zeiss). Images were processed (cropping, brightness and contrast adjustments to entire image) using Adobe Photoshop CS4/CS5 and/or Zen 2008/9/11.

Quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) according to manufacturer's instructions, DNase (Fisher Scientific) treated and cleaned using an RNA clean up kit (Zymo) before reverse transcription (iScript, Bio-Rad). Prior to RNA extraction, animals were starved for 7 days after the last RNAi feeding to ensure that any remnant dsRNA was cleared from the system. qRT-PCR was performed using GoTaq qPCR master mix (Promega) using Applied Biosystems StepOne Plus RT-PCR system. All experiments were done in biological and technical triplicates. Transcript levels were normalized to *β -tubulin*. Relative mRNA levels were calculated using $\Delta\Delta CT$. All primers are listed in SI Appendix, Table S4.

Supplementary Figure Legends

Figure S1. Planarian *boule* homologs are expressed in the ovaries. Colorimetric in situ hybridization for *boule1* and *boule2* showing expression in the female reproductive system. *boule1* expression was seen in the ovaries of some animals (n=3/8) and possible *boule2* expression was detected in the ventral portion of the animals (n=7/7), where the ovaries are located. Scale bars, 100 μ m.

Figure S2. *boule1* and *boule2* are expressed in spermatogonial stem cells (SSCs) and spermatogonia in the male germline. Double fluorescence in situ hybridization (FISH) showing (A) *boule1* and (B) *boule2* coexpressed with *nanos*, which labels SSCs. Scale bars, 50 μ m. Magnified sections showing colocalization of (A') *boule1* and (B') *boule2* with *nanos* (arrows). *nanos*⁺ cells show lower intensity of *boule1* signal than the surrounding spermatogonia. Scale bars, 10 μ m. (C) *boule1* and (D) *boule2* are coexpressed with *germinal histone H4 (gH4)* transcript in the male gonads. *gH4* labels SSCs and spermatogonia in the male germline. Scale bars, 50 μ m.

Figure S3. Effect of *boule1* or *boule2* RNAi on SSCs and spermatids (A) Distinct stages of planarian spermatogenesis and labels for individual testis cell types. *nanos* and *germinal histone H4* (in magenta) label SSCs and spermatogonia, respectively. *tektin-1* and *protein kinase A* (in cyan) label spermatocytes and spermatids respectively. (B) Following 2 feedings of *boule1* and *boule2* dsRNA (intermediate knockdown), *boule1*(RNAi) animals have SSCs (labeled by *nanos*) similar to controls (n=6/6). Half (n=3/6) the *boule2*(RNAi) animals show *nanos* expression and the remaining 3 animals have no *nanos*⁺ SSCs. The spermatid population, labeled with *pka*, is slightly reduced in *boule1*(RNAi) animals, and the *gH4*⁺ spermatogonial population is expanded (n=5/5). *boule2*(RNAi) animals show *pka* labeling

comparable to controls (n=6/6). **(C)** At later stages, following 4 feedings of dsRNA *boule1(RNAi)* animals have testes with clusters of SSCs and spermatogonia, and animals lack meiotic and postmeiotic cells (n=5/5). *boule2(RNAi)* animals show a complete loss of all male germ cells (n=6/6). Scale bars, 50 μ m.

Figure S4. Demonstration of *boule1* or *boule2* RNAi specificity. **(A)** Alignment of *boule1* and *boule2* nucleotide sequences showing no significant similarity between the sequences. **(B)** and **(C)** show qRT-PCR validation of *boule1* or *boule2* knockdown following a single dsRNA feeding. **(B)** Following *boule1(RNAi)* there is an increase in *boule2* transcript, most likely due to accumulation of spermatogonia, in which *boule2* is expressed. **(C)** The levels of *boule1* transcript are similar to controls in *boule2(RNAi)* animals. Two-tailed unpaired t-test with Welch's correction was performed for all samples, $P < 0.05$. **(D)** FISH for *boule1* or *boule2* was performed following 2 dsRNA feedings of either gene. Our experiments show that the knockdown of either *boule1* or *boule2* does not affect the expression of the other paralog. Scale bars, 50 μ m.

Figure S5. *boule2(RNAi)* animals show increased apoptosis. TUNEL was performed on cryosections following 2 feedings of *boule1* or *boule2* dsRNA. **(A)** *boule2(RNAi)* animals show a greater number of TUNEL⁺ cells in the testes compared to control and *boule1* knockdown animals. Scatter plot shows mean with standard deviation. One-way ANOVA was performed using Dunnett's multiple comparisons test to determine significance at 95% confidence interval. **(B)** Representative images showing TUNEL⁺ cells (arrows) in *boule2(RNAi)* animals. Scale bars, 20 μ m.

Figure S6. Validation of *boule1* and *boule2* gene knockdowns in regenerates and hatchlings (A)

dmd1(RNAi) head fragments do not respecify their SSCs 14 days post amputation (n=4/4). Scale bars, 100 μ m. **(B)** qRT-PCR for samples corresponding to Figures 2A and B. Relative mRNA levels of *boule1* and *boule2* are low after 14 days of regeneration. Error bars represent 95% confidence intervals calculated based on standard error of the mean. Two-tailed unpaired t-test with Welch's correction was performed for all samples, $P < 0.05$. **(C,D)** Knockdown phenotype of *boule1* or *boule2* in sexual hatchlings (<48 hours old) is similar to the RNAi phenotype in adults. **(C)** Following two feedings of *boule1* dsRNA, *nanos* and *gH4* expression appears comparable to control animals (n=6/6). Some *boule2(RNAi)* animals (n=2/6) show absence of male germ cells, and the remaining animals (n=4/6) show very small testis lobes with both *nanos*⁺ and *gH4*⁺ germ cells. **(D)** After 4 feedings of *boule1* dsRNA, animals show accumulation of spermatogonia compared to controls, and *boule2(RNAi)* animals lack all male germ cells. Scale bars, 50 μ m.

Figure S7. Assay for determining the role of *boule1* and *boule2* in male germline regeneration and

differentiation. (A) Experimental schematic. When planarians are amputated posterior to their ovaries, the resulting tail fragments regress their testes approximately 7 days post-amputation, and contain clusters of early, undifferentiated male germ cells. Tail regenerates were fed *boule1* or *boule2* dsRNA (4 feedings, 4-5 days apart) after amputation and testes regression. **(B)** Testes were restored in control regenerates (n=6/6). *boule1(RNAi)* regenerates had small testis lobes containing only SSCs and spermatogonia (n=5/6). By contrast, in *boule2(RNAi)* regenerates, all the male germ cells were absent (n=6/6). **(C)** qRT-PCR validation. Amputated animals at the beginning of RNAi show low levels of *tkn-1* (spermatocytes) and *pka* (spermatids). Error bars represent 95% confidence intervals calculated based

on standard error of the mean. Two-tailed unpaired t-test with Welch's correction was performed for all samples, $P < 0.05$.

Figure S8. Additional experiments with putative planarian DAZ family-associated proteins. (A)

Control (RNAi) animals show the expression of all four germ cell markers. **(B)** *DAZAP2* is not required for the maintenance of the male germ cells in homeostasis ($n=6/6$). **(C)** *DAZAP1(RNAi)* and **(D)** *iguana(RNAi)* in homeostasis results in no change in SSC (*nanos*⁺) population and an accumulation of rounded spermatids (*pka*⁺) population ($n=6/6$ for both). **(E-F)** *DAZAP1(RNAi)* in sexually immature regenerates corroborates the gene's homeostasis phenotype, with animals showing no mature sperm ($n=4/6$). *iguana(RNAi)* animals undergo lysis upon amputation ($n=6/6$, Table S1). Scale bars, 50 μm .

Figure S9. Additional experiments on putative planarian DAZ family targets. (A) Planarian

homologs of DAZ/DAZL targets are expressed in the male germline. Scale bars, 1mm. **(B-D)** *SDAD1(RNAi)*, *CDC25-1(RNAi)* and *CDC25-2(RNAi)* have SSCs (*nanos*) and spermatids (*pka*) ($n=6/6$ for all except *SDAD1*) at early stages of knockdown. Half ($n=3/6$) of *SDAD1(RNAi)* animals show no *nanos* labeling. *CDC25-1* knockdown results in enlarged SSCs possibly due to defects in cytokinesis (see insets in B and D). Sexually immature regenerates fed **(E)** control dsRNA regenerate their testes, whereas **(F)** in the absence of *CDC25-2*, regenerates cannot maintain the early male germ cells, similar to *boule2(RNAi)* animals ($n=6/6$). *SDAD1(RNAi)* and *CDC25-1(RNAi)* animals undergo lysis upon amputation ($n=6/6$ for both, Table S2). Scale bars, 50 μm ; inset scale bars, 10 μm .

Figure S10. Phylogenetic analyses of the DAZ family (A) Alignment of Boule, DAZL, and DAZ

RRMs. **(B)** Branch length ratios of pre-meiotic (Smed-Boule2 or Dazl) to meiotic (Smed-Boule1 or

vertebrate Boule) terminal edge lengths in planarians and vertebrates. Note the markedly similar distribution of paralog branch length ratios in species with neofunctionalized Boule derivatives.

Table S1. Experimental details for planarian homologs of DAZ-associated proteins

Table S2. Experimental details for planarian homologs of DAZ family targets

Table S3. Accession numbers of sequences used for phylogenetic analyses

Table S4. Cloning and qRT-PCR primer sequences

Figure S1

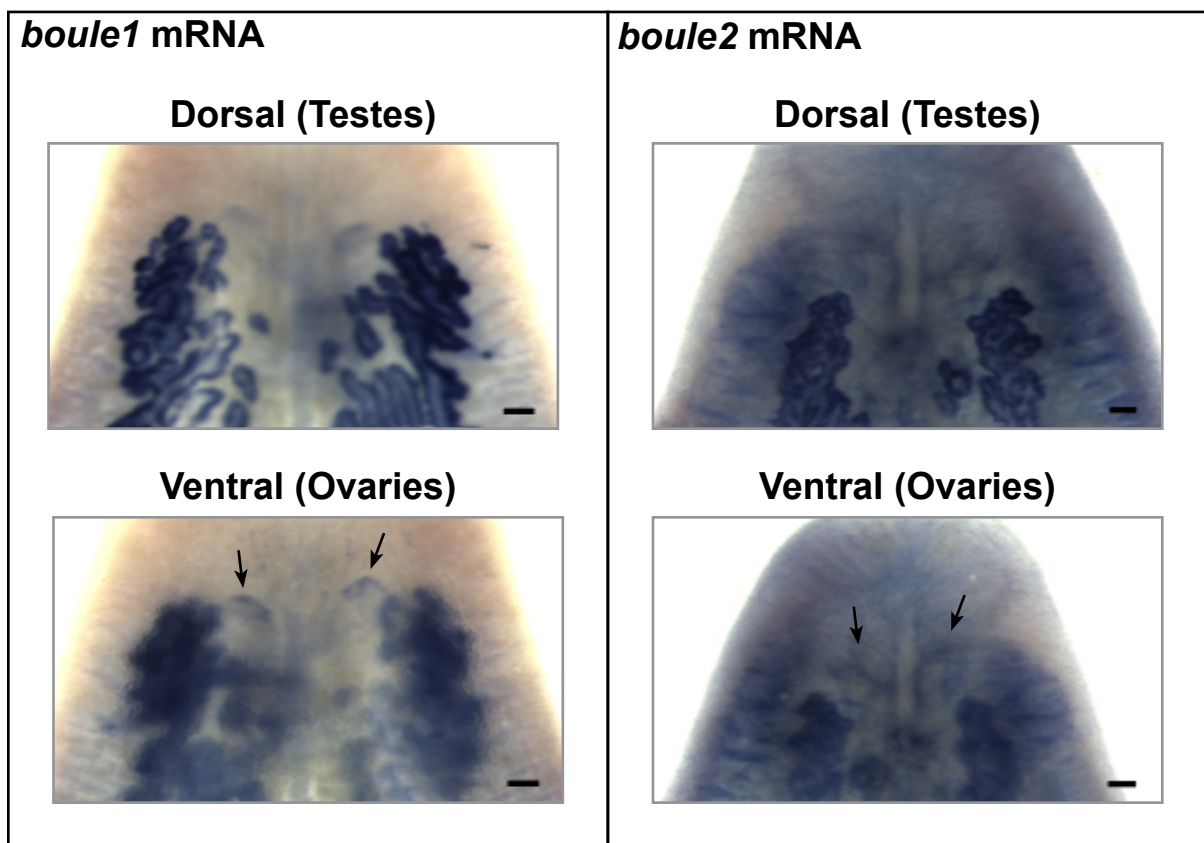


Figure S2

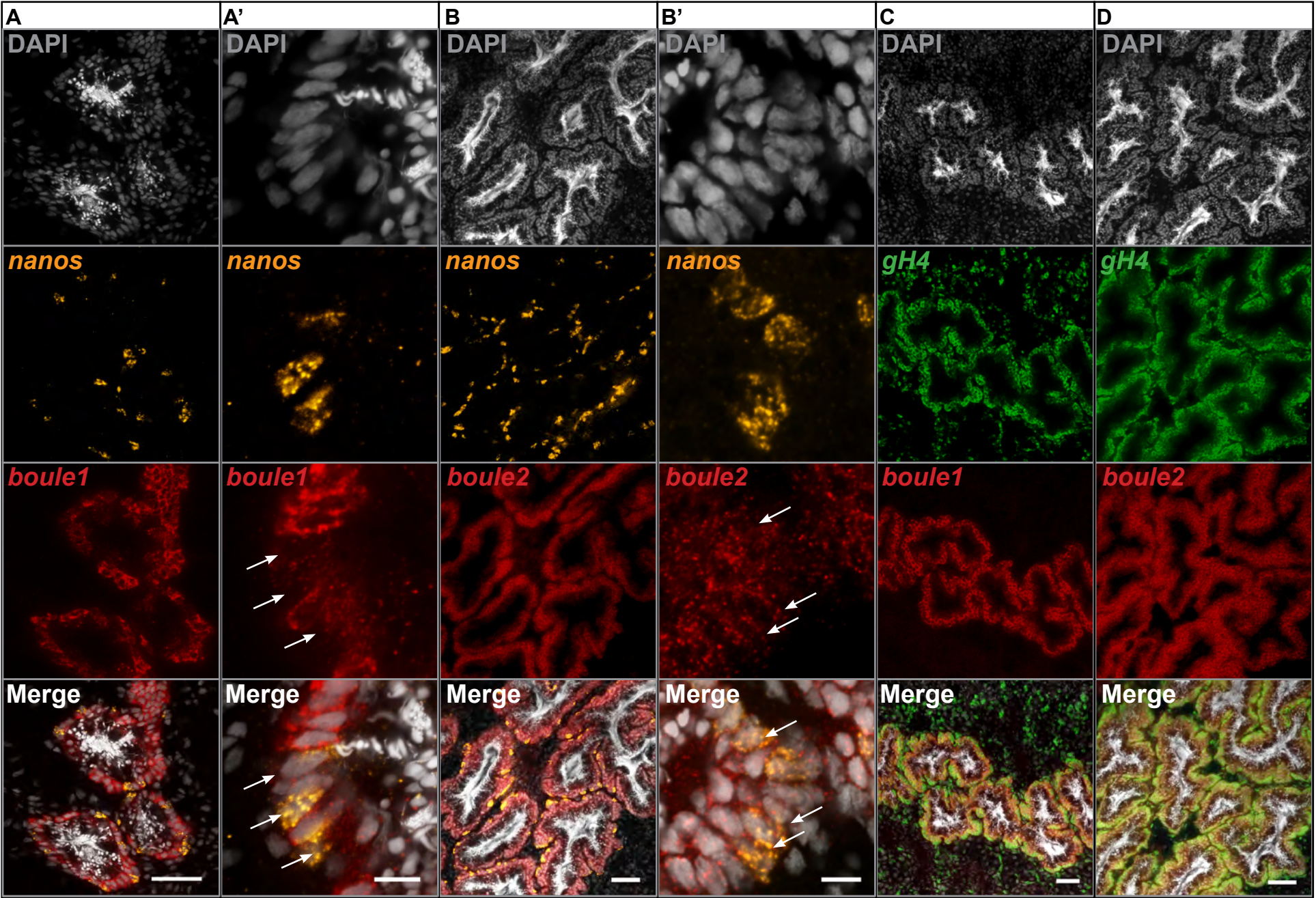


Figure S3

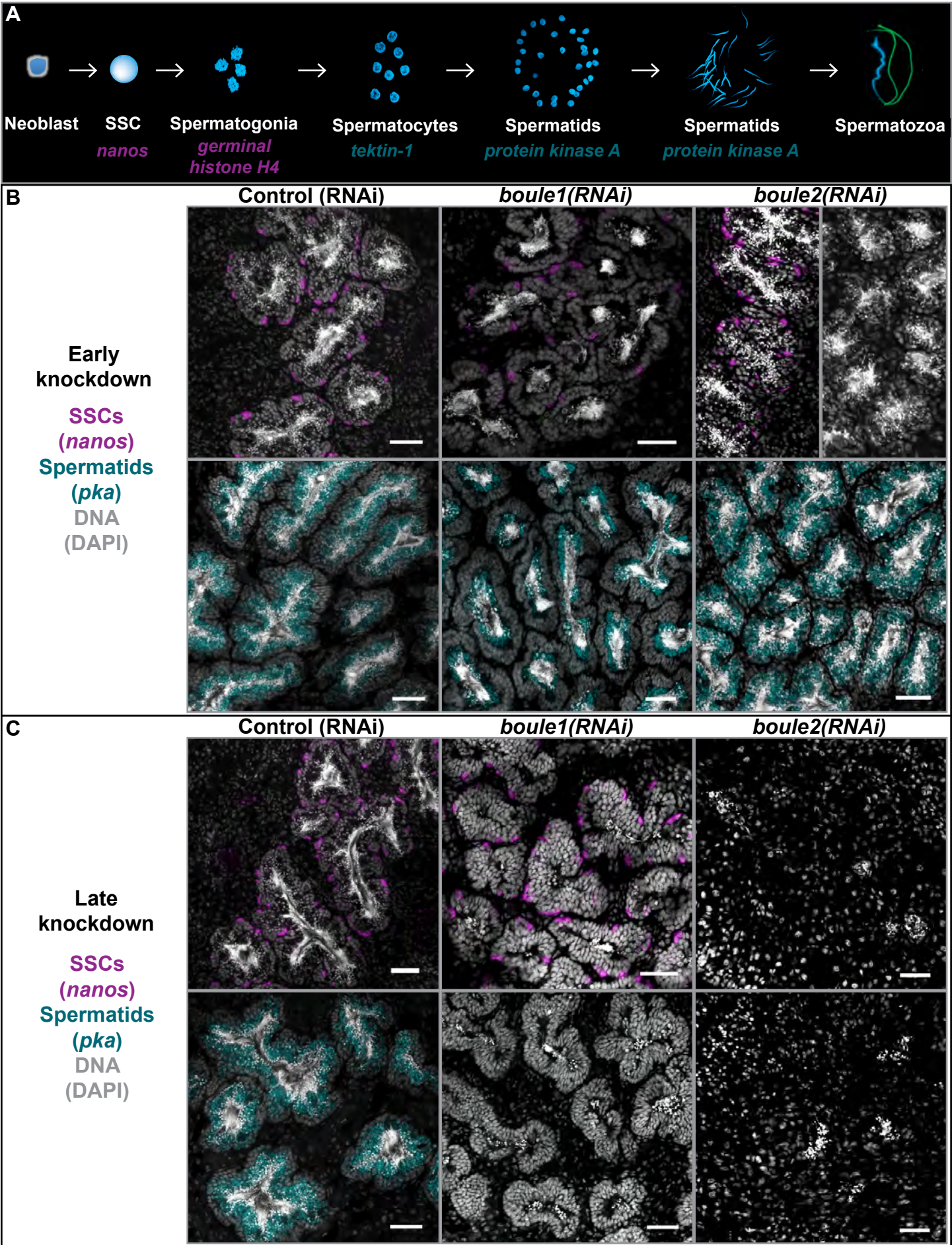
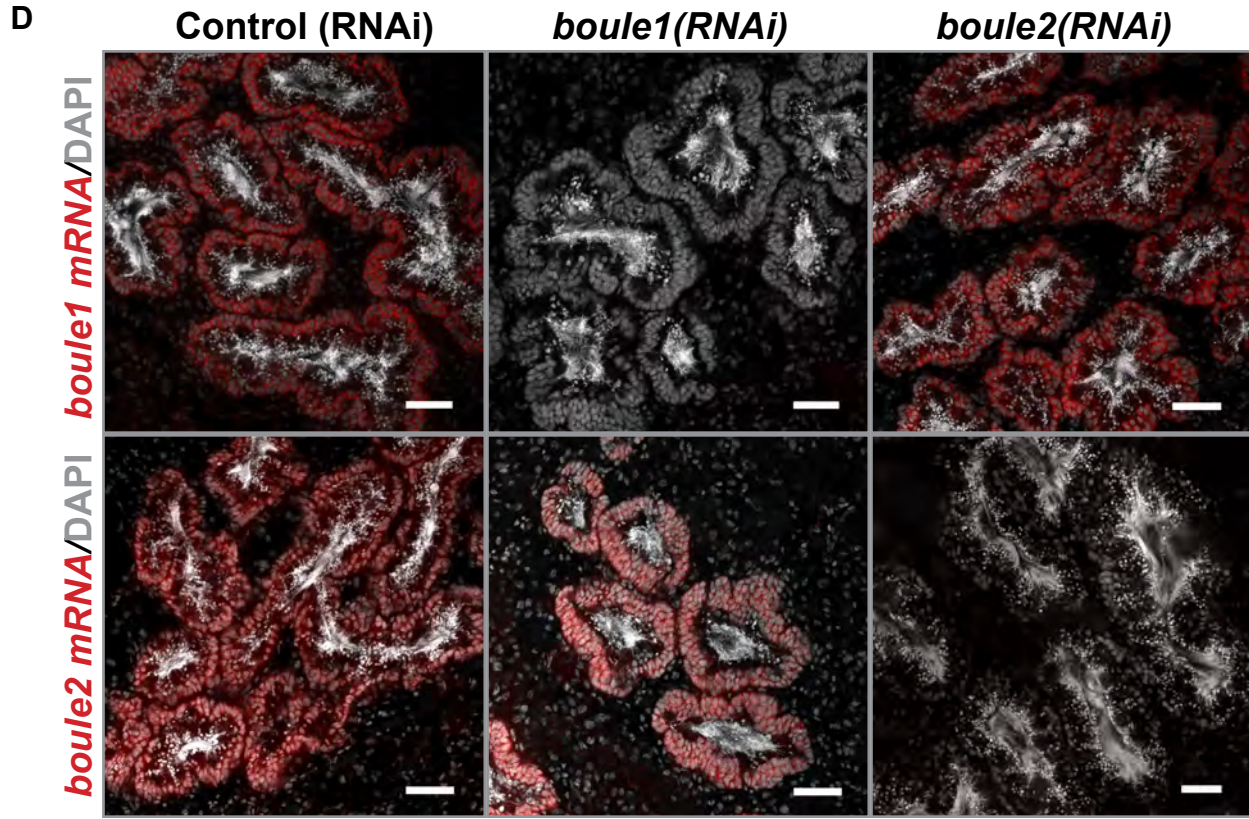
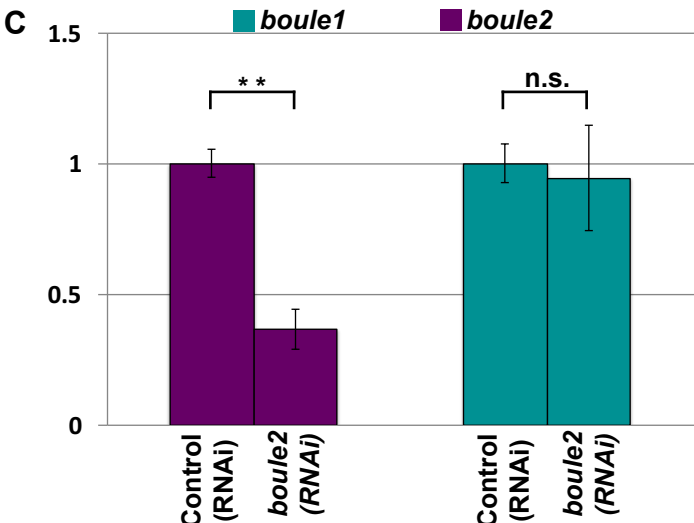
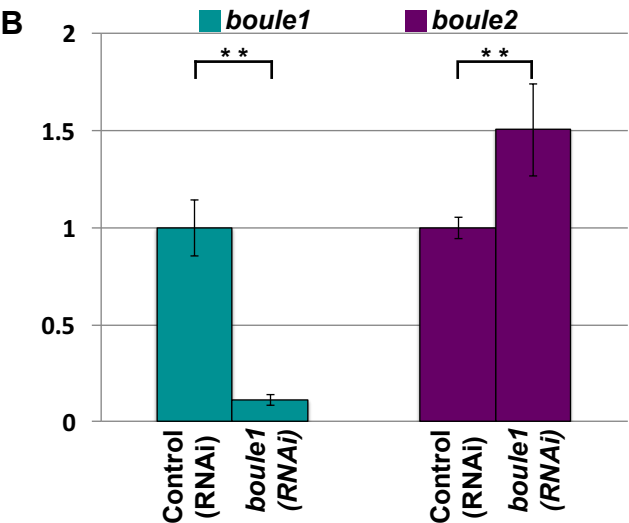
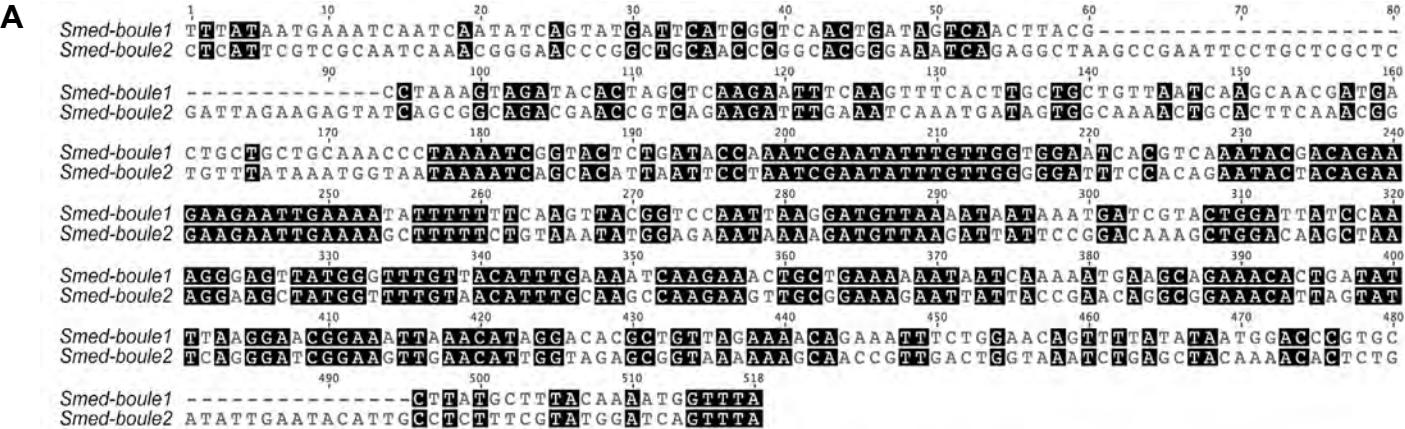


Figure S4



A

Number of TUNEL⁺ cells per testis lobe

Control (RNAi) *boule1* (RNAi) *boule2* (RNAi)

n.s. **

Condition	Number of TUNEL ⁺ cells per testis lobe (individual data points)
Control (RNAi)	1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 2.0, 2.0, 3.0, 3.0
<i>boule1</i> (RNAi)	1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 2.0
<i>boule2</i> (RNAi)	0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 2.0, 2.0, 2.0, 2.0, 2.0, 2.0, 3.0, 3.0, 3.0, 3.0, 4.0, 4.0

Control (RNAi)

boule1(RNAi)

boule2(RNAi)

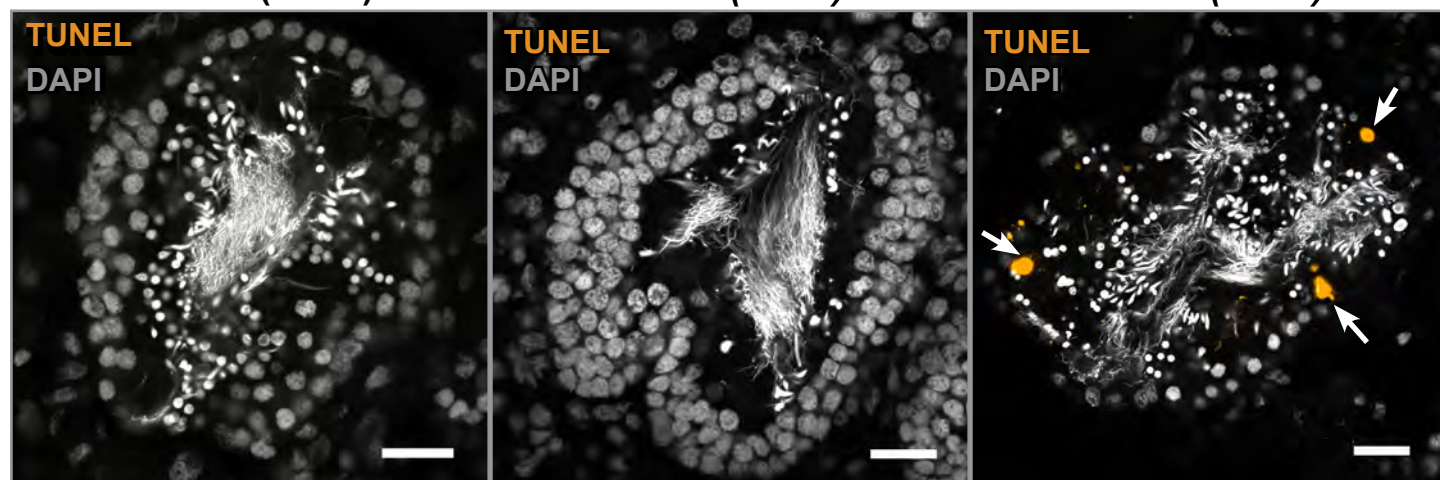


Figure S6

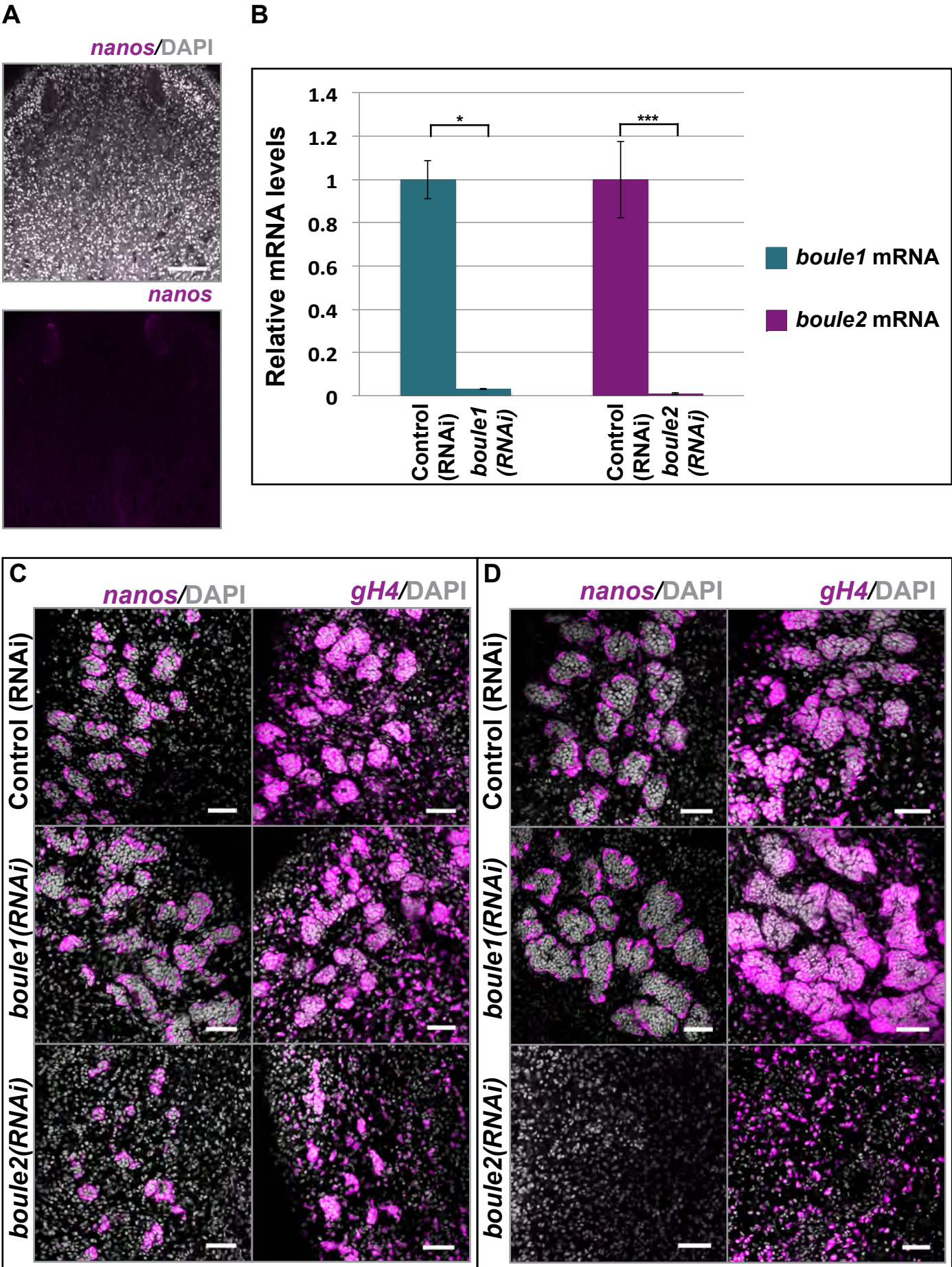


Figure S7

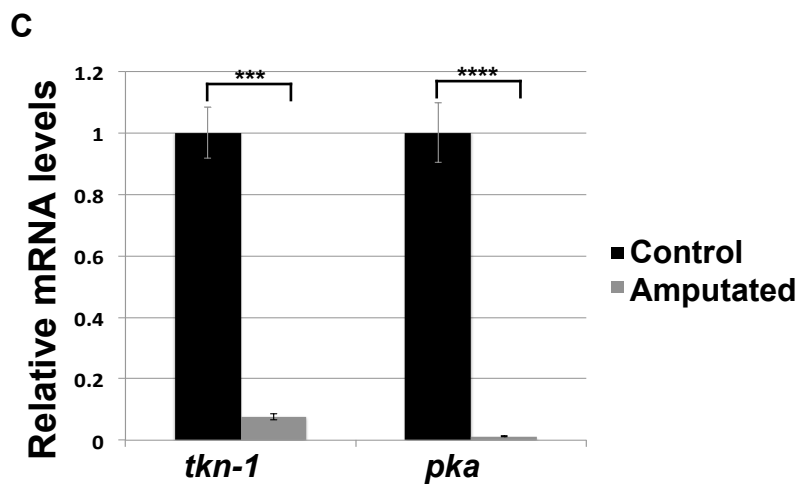
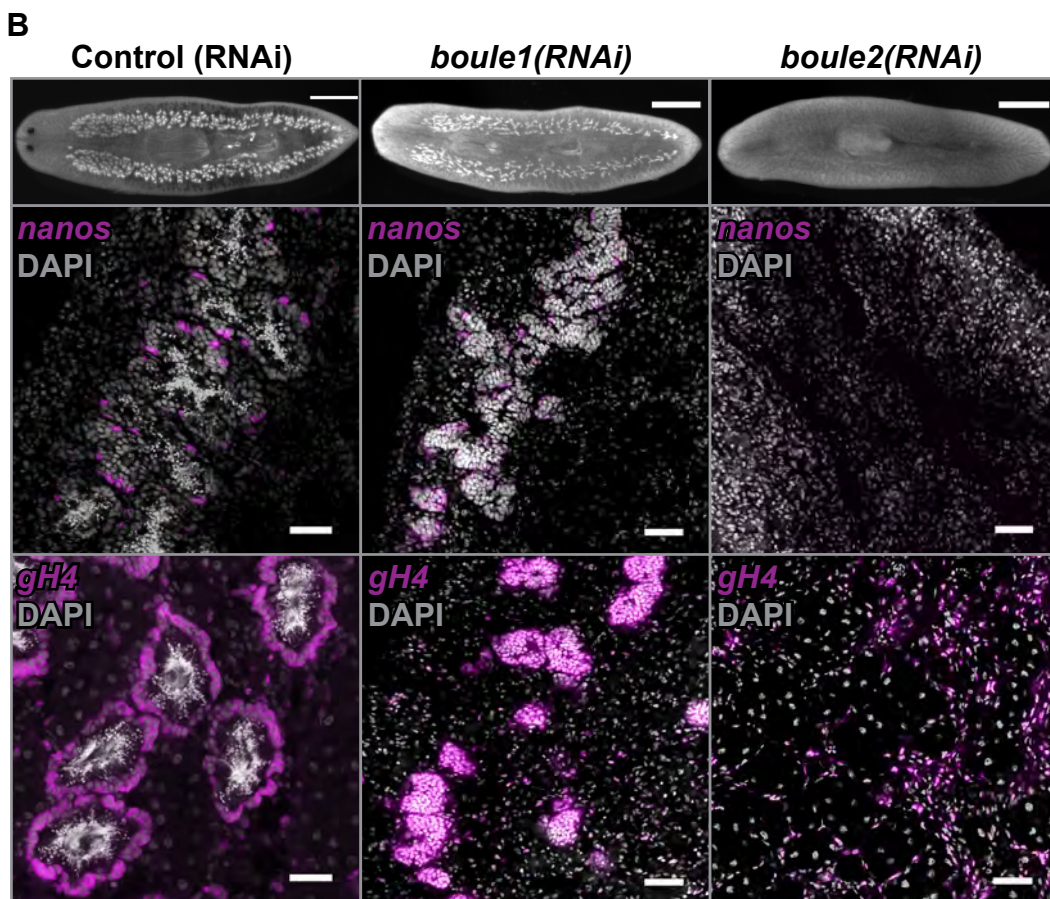
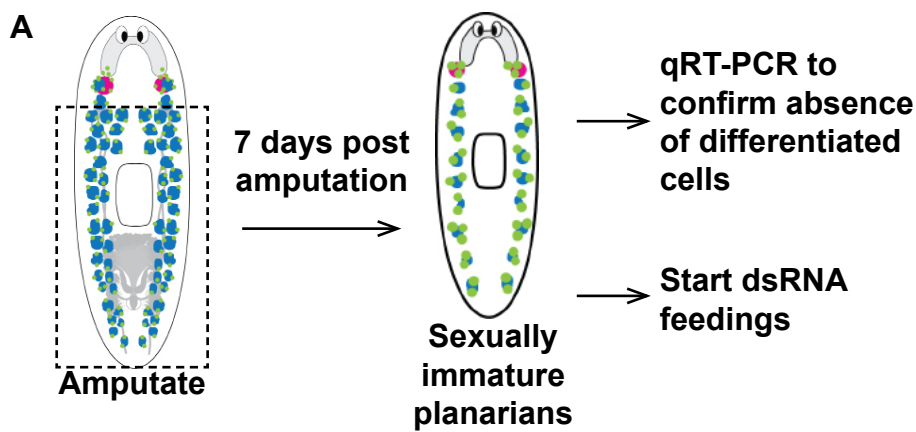


Figure S8

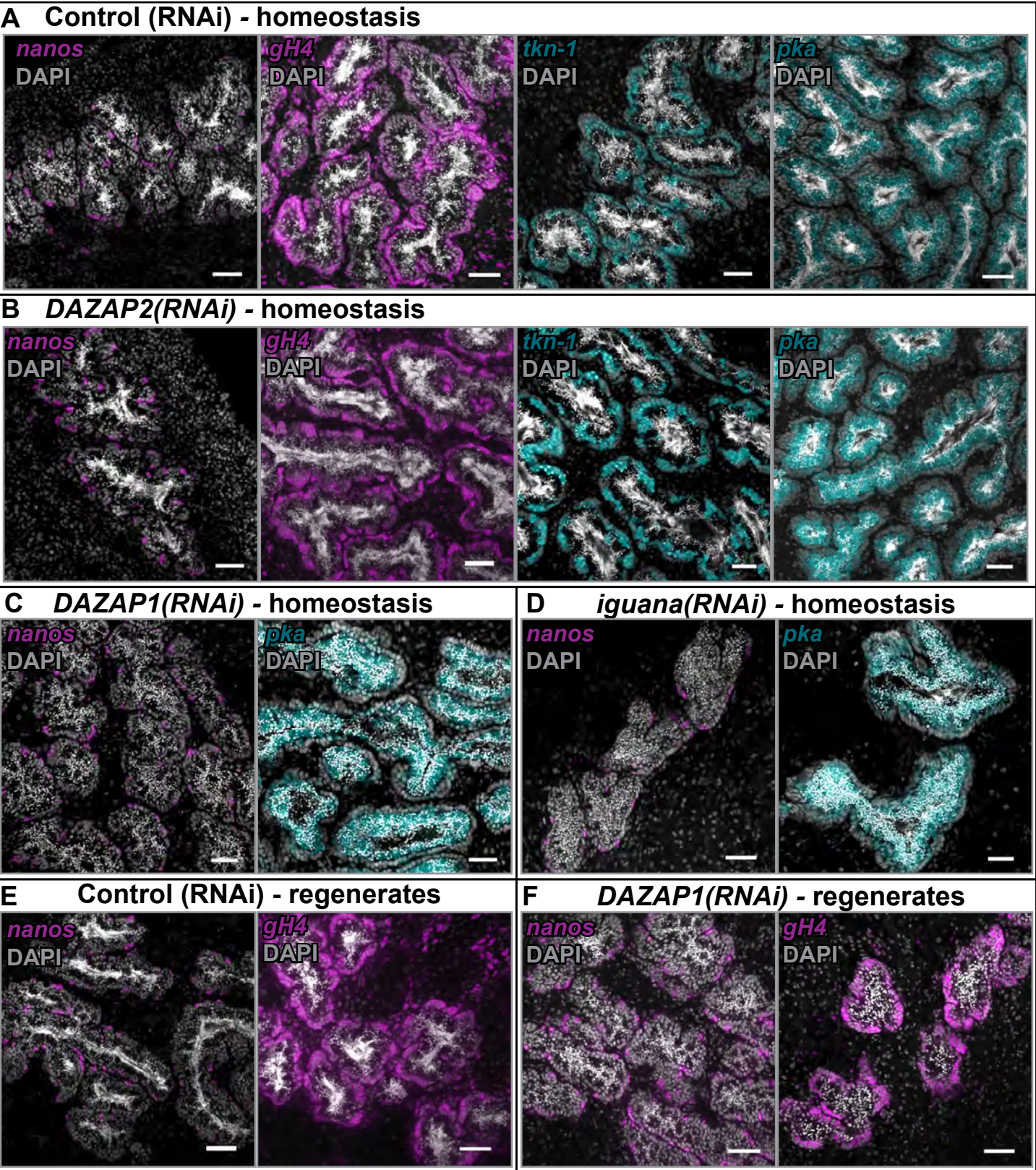
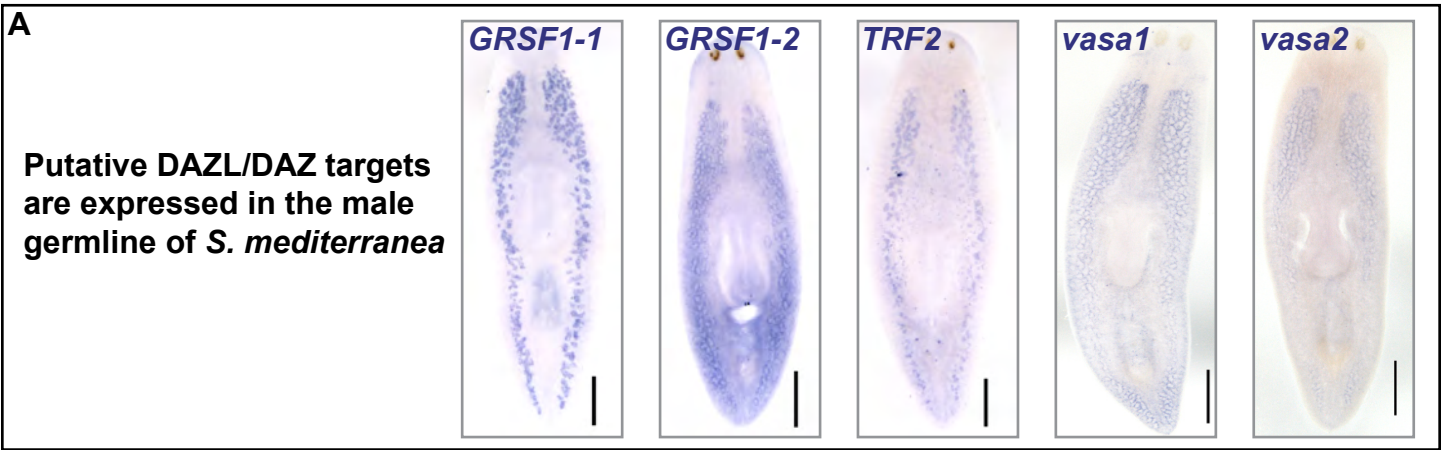
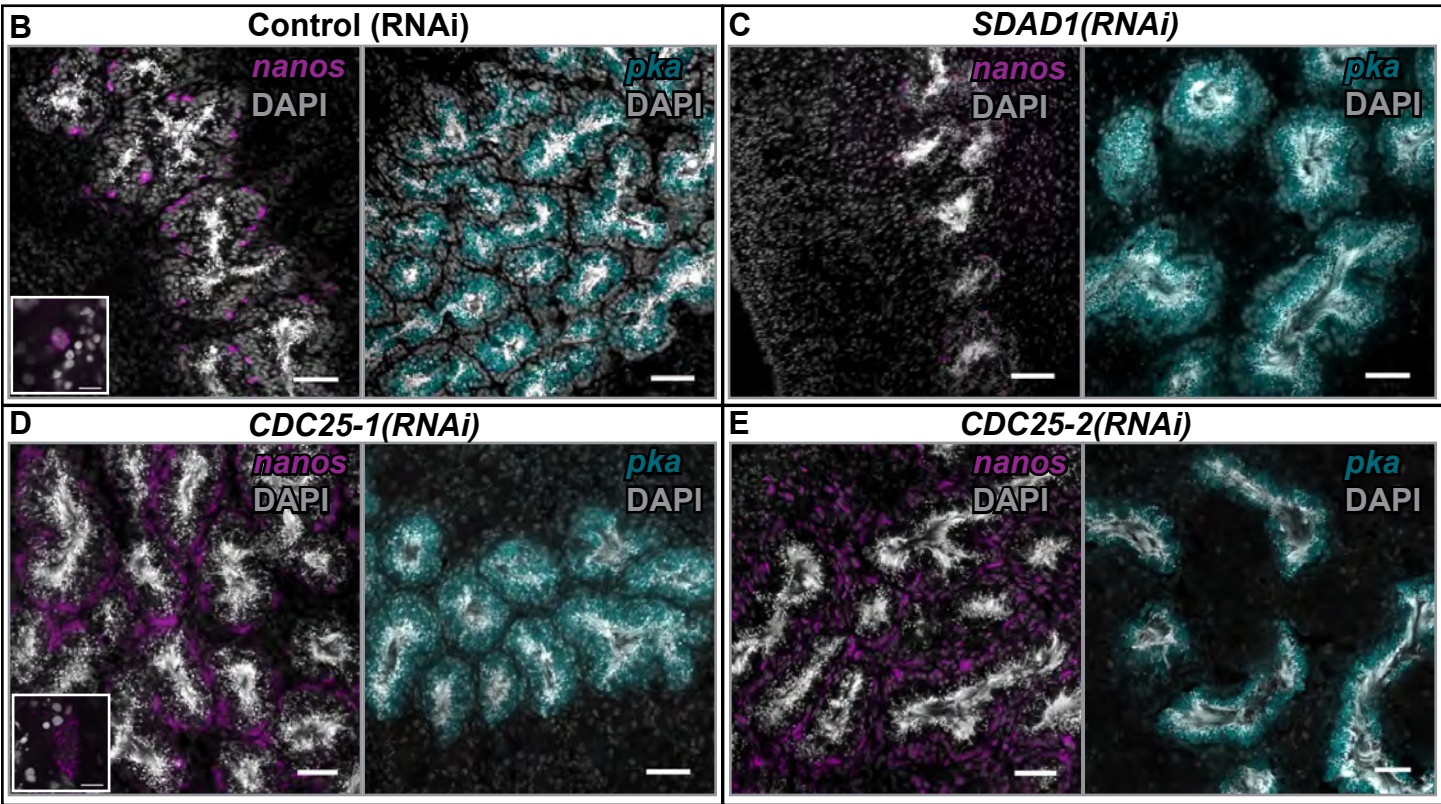


Figure S9



SSCs and spermatids in early stages of *SDAD1*, *CDC25-1*, and *CDC25-2* knockdown



CDC25-2(RNAi) and *boule2*(RNAi) in sexually immature regenerates phenocopy each other

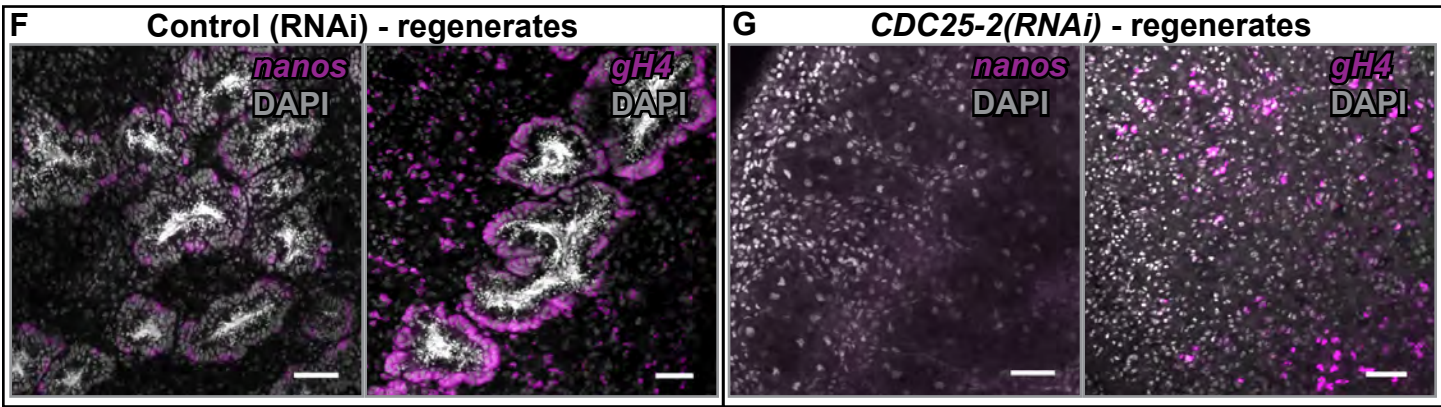


Figure S10

A

<i>S. cerevisiae</i> HRP1	KADLSKESCK	MFIGGLNWD	TTEDNLREYF	GKY---	GTVT	DLKIMKDPAT	GRSRG-	FGFL
<i>H. vulgaris</i> Boule2	VIYGTHYPNR	LFVGCLPPD	AGAEDLGTFE	ANY---	GNVV	EAKVVLDK-F	GVSQR-	FGFV
<i>N. vectensis</i> Boule1	FLLGKRYPNK	IFVGGLPPL	TTAEELANFF	ANF---	GHVV	ESKVIFDH-D	GISKG-	YGFV
<i>H. miamia</i> Boule1	SKSHVLIPNR	IFLGLSLS	VFESDIEAFF	AEF---	GAVN	DVKIIRKE--	GMDRA-	YGFV
<i>H. vulgaris</i> Boule3	SPGDVEIPNR	IFVKGFSKE	TTEGDLINFF	EIY---	GIVI	EANIIRDK-Q	GLSKG-	YGFV
<i>N. vectensis</i> Boule2	DVPVLRYPNR	IFVGGVAFO	TTAIELRELF	ESY---	GAVR	DVKIARDG-E	GVSQR-	YGFV
<i>H. miamia</i> Boule2	YVTGALVPNS	VFVGGIAPN	ITDIEIANYP	SLIATKGVN		EVKIIIFDK-D	GSNKG-	YCFV
<i>H. vulgaris</i> Boule1	PDGGLIIPNR	VFLGGIPTE	TTELELELFF	SDY---	GLVK	DVRIIVTDRVT	GECKG-	YGFV
<i>M. lignano</i> Boule1	SATETVIENR	IFIGGIPPH	ADENEFRNFL	TSTFDKFMK		DVKIIFDR-T	GASKG-	YGFV
<i>M. lignano</i> Boule2	POLETVIENR	IFVGGIPSH	ANVSELRAFL	AEKFNKAEIK		DIKIIISDK-T	GVSQR-	YGFV
<i>X. laevis</i> Daz1	LPEGKIMPNT	VFVGIDIT	MDEIEIRDFE	TRF---	GNVK	EVKIIITDR-T	GVSQR-	YGFV
<i>G. gallus</i> Daz1	LPEGKIMPNT	VFVGIDIR	MNEAEIRSYF	EQY---	GTVK	EVKIIITDR-T	GVSQR-	YGFV
<i>H. sapiens</i> DAZ	LPEGKIVPNT	VFVGIDAR	MDETEIGSCF	GRY---	GSVK	EVKIIITNR-T	GVSQR-	YGFV
<i>M. musculus</i> Daz1	LPEGKIMPNT	VFVGIDVR	MDETEIRSFF	ARY---	GSVK	EVKIIITDR-T	GVSQR-	YGFV
<i>H. sapiens</i> DAZL	LPEGKIMPNT	VFVGIDVR	MDETEIRSFF	ARY---	GSVK	EVKIIITDR-T	GVSQR-	YGFV
<i>D. rerio</i> Daz1	LPEGKMTPTNT	LFVGGIDMK	VDENEIREFF	AKY---	GSVK	EVKIIITYR-G	GICKG-	YGFV
<i>O. latipes</i> Daz1	LPEGKLTPTNS	LFVGGIDMK	VDENEIKEFF	ARY---	GSVR	EVKIIIRYR-G	GLCKG-	YGFV
<i>C. elegans</i> Boule(Daz1)	PPTYELIPNR	IFVGGFPTS	TTETELREHF	EKF---	FAVK	DVKMVKSL-D	GQSKG-	YGFV
<i>C. briggsae</i> Boule	PPTYELIPNR	IFVGGFPTS	TTESELDRHF	ERF---	YPVK	DVKMVKSL-D	GHSKG-	YGFV
<i>H. robusta</i> Boule2	PPTYGLVLPNR	IFVGGIGGD	VTESDLKSFF	SSY---	GPIR	HCKIMLDN-N	GMSKG-	YGFV
<i>M. lignano</i> Boule3	ELGGTLIPNR	VFVGIPSS	ATEAELMEFF	SAL---	GEVR	DVKIIADK-S	GTSKG-	YGFV
<i>P. fucata</i> Boule	PKYGTLPVNR	VFVGIIAAN	TTEQELKQFF	TGY---	GAVK	DTKIIIVDR-A	GVSQR-	YGFV
<i>D. melanogaster</i> Boule	PKYGTLPVNR	IFVGGISGD	TTEADLTRVF	SAY---	GTVK	STKIIIVDR-A	GVSQR-	YGFV
<i>D. ananassae</i> Boule	PKYGTLPVNR	IFVGGISGD	TTEADLTRVF	SAY---	GTVK	STKIIIVDR-A	GVSQR-	YGFV
<i>T. castaneum</i> Boule	PKYGTLPVNR	IFVGGISAN	TTEGELLQFF	SSY---	GTVK	AAKIIQDR-A	GVSQR-	YGFV
<i>S. kowalevskii</i> Boule	PRFGTLIPNR	IFVGGITYD	TNDSALKEYF	SKF---	GHVK	EAKIICDR-A	GVSQR-	YGFV
<i>G. gallus</i> Boule	PRFGTVIPNR	IFVGGIDFK	TNENDLRKFF	AQY---	GCVR	EVKIVNDR-A	GISKG-	YGFV
<i>M. musculus</i> Boule	PRYGTVIPNR	IFVGGIDFK	TNENDLRKFF	SOY---	GSVK	EVKIVNDR-A	GVSQR-	YGFV
<i>H. sapiens</i> Boule	PRYGTVIPNR	IFVGGIDFK	TNESDLRKFF	SOY---	GSVK	EVKIVNDR-A	GVSQR-	YGFV
<i>S. mediterranea</i> Boule2	NKISTLIPNR	IFVGGFPQN	TTEEELKSFF	CKY---	GEIK	DVKIIPDK-A	GQAKGS	YGFV
<i>S. purpuratus</i> Boule	PRYGTIIPNR	IFVGGIAFNO	TSDAELRNFF	SAF---	GHVK	EAKIADR-A	GVSQR-	YGFV
<i>H. robusta</i> Boule1	PKFGTLIPNR	VFVGIISSD	TSENELKFFF	SAY---	GPVK	DKKIILDR-G	GISKS-	YGFV
<i>Capitella</i> Boule1	PKYGTLPVNR	IFVGGISGN	ATEAELKQFF	TQF---	GAVK	DAKIIIMDR-A	GVSQR-	YGFV
<i>Capitella</i> Boule2	PKYGTLPVNR	IFVGGISGN	ATEAELKQFF	TQF---	GAVK	DAKIIIMDR-A	GVSQR-	YGFV
<i>S. mediterranea</i> Boule1	PKIGTLIPNR	IFVGGITSN	TTEEELKNFF	SSY---	GPIK	DVKIINDR-T	GLSKGS	YGFV
<i>S. mansoni</i> Boule	PKFGTLIPNR	IFVGGIPSN	TNEQELKSFF	SSF---	GHVK	DVKIINDR-L	GASKG-	YGFV
<i>E. multilocularis</i> Boule1	PKFGTLIPNR	IFVGGIPSN	TTEQELKTYF	SSF---	GQVK	DVKIINDR-L	GVSQR-	YGFV
<i>H. microstoma</i> Boule	PKIGTLIPNR	IFVGGIPSN	TTEQELKAYF	SSF---	GQVK	DVKIINDR-L	GVAKGS	YGFV
<i>H. diminuta</i> Boule1	-----	-----	TTEQELKAYF	SSF---	GQVK	DVKIINDR-L	GVAKGS	YGFV
<i>O. bimaculoides</i> Boule	PKYGTVIPNR	IFVGGITTT	TTEAELKAFF	TSY---	GTVK	DTKIIADR-G	GVSQR-	YGFV
<i>L. anatina</i> Boule	PKYGTVIPNR	IFVGGIAPN	TTEQELRHYF	SAF---	GAVK	DTKIIADR-A	GVSQR-	YGFV
<i>L. gigantea</i> Boule	PKFGTVIPNR	IFVGGIAAN	TTDAELKQFF	SAY---	GAVK	DTKIIADR-G	GVSQR-	YGFV
<i>A. californica</i> Boule	PKYGTIIPSR	IFVGGIAAN	TTDAELKQYF	SAF---	GAVK	DTKIIITDR-A	GVSQR-	YGFV
<i>B. glabrata</i> Boule	PKFGTVIPSR	IFVGGIAAN	TTDAELKQYF	SAF---	GAVK	DTKIIITDR-A	GVSQR-	YGFV
<i>H. diminuta</i> Boule2	PKIGTIIPNR	IFVGGISSN	TTEDDLRKFF	EKY---	GHVK	DVKIIFDH-S	ILSKGN	YGFV
<i>E. multilocularis</i> Boule2	PKIGTLIPNR	IFVGGITST	TTEDDLRTFF	GKF---	GQVK	DVKIISDR-S	GLSKGN	YGFV

B

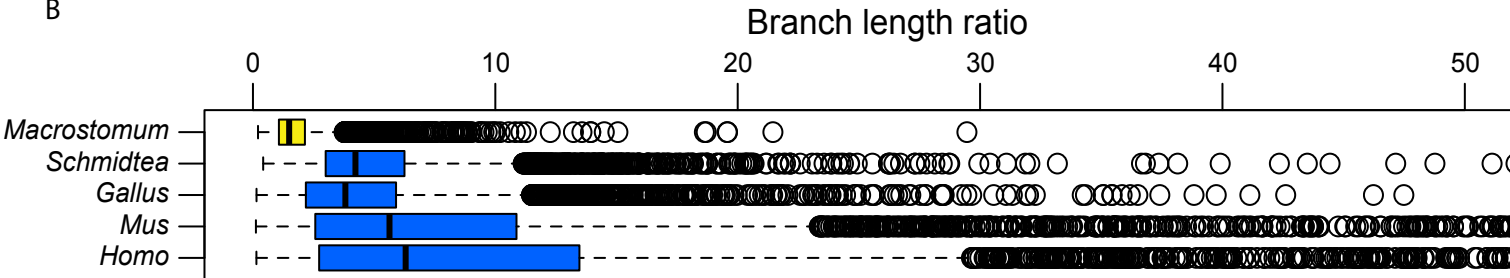


Table S1. Experimental details for planarian homologs of DAZ-associated proteins

Associated protein name	Required for regeneration	Expressed in male germ cells?	Germ cell RNAi phenotype in adult sexually mature worms	Germ cell RNAi phenotype in hatchlings	Required for <i>de novo</i> specification of germ cells?	RNAi phenotype in sexually immature regenerates
<i>DAZAP1</i>	No	Yes	No mature sperm	No mature sperm	No	No mature sperm
<i>DAZAP2</i>	No	Yes	No phenotype	No phenotype	No	Small testes with only SSCs and spermatogonia
<i>DAZAP1+2</i>	No	-	No mature sperm	Not tested	Not tested	Not tested
<i>DZIP/Smed-iguana</i>	Yes (bloating observed in homeostasis)	Yes	No mature sperm	Not tested	Lysis	Lysis, no testes in remaining fragments
<i>pumilio</i>	Yes	Yes	Small testes, no loss of specific cell type marker	Not tested	Lysis	Lysis, no testes in remaining fragments

Table S2. Experimental details for planarian homologs of DAZ family targets

Target name	Required for regeneration (neoblast maintenance)?	Expressed in the male germ cells?	Germ cell RNAi phenotype in adult sexually mature worms	Germ cell RNAi phenotype in hatchlings	Required for <i>de novo</i> germ cell specification?	RNAi phenotype in sexually immature regenerates
<i>CDC25-1</i>	Yes	Yes	Loss of early germ cells followed by more differentiated cells	No male germ cells	Lysis	Lysis, no testes in remaining fragments
<i>CDC25-2</i>	No	Yes	Loss of early germ cells followed by more differentiated cells	No male germ cells	No	No testes
<i>SDAD1</i>	Yes	Yes	Loss of early germ cells followed by more differentiated cells	Not tested	Lysis	Lysis
<i>CDC25-3</i>	No	Not tested	No	Not tested	No	No
<i>vasa1</i>	Yes	Yes	Small testes, no loss of specific cell type marker	Not tested	Lysis	Lysis
<i>vasa2</i>	No	Yes	No	Not tested	No	Small testes with only SSCs and spermatogonia
<i>Ringo/SPY</i>	No	Yes	No mature sperm	Not tested	No	Small testes with only SSCs and spermatogonia
<i>TPX-1</i>	No	No	No	Not tested	No	No
<i>TRF2-1</i>	No	Yes	No	Not tested	No	No
<i>TRF2-2</i>	No	No	No	Not tested	No	No
<i>TRF2-3</i>	No	No	No	Not tested	No	No
<i>GRSF1-1</i>	Yes	Yes	Early lysis	Not tested	Lysis	Lysis
<i>GRSF1-2</i>	No	Yes	No	Not tested	No	No
<i>PAM</i>	No	Not tested	No	Not tested	No	No
<i>TSSK</i>	No	Not tested	No	Not tested	No	No

Table S3. Accession numbers of sequences used for phylogenetic analyses

Organism	Common name/taxon	Gene name	NCBI Accession number/source
<i>Aplysia californica</i>	Mollusc	Boule	XP_005103136
<i>Caenorhabditis elegans</i>	Nematode	Boule	NM_062635.4
<i>Caenorhabditis briggsae</i>	Nematode	Boule	XP_002630720
<i>Drosophila ananassae</i>	Arthropod (Diptera)	Boule	XP_001958310
<i>Drosophila melanogaster</i>	Arthropod (Diptera)	Boule	Q24207
<i>Gallus gallus</i>	Aves	Boule	XP_421917
<i>Hofstenia miamia</i>	Acoelomorpha	Boule1	Not available
<i>Hofstenia miamia</i>	Acoelomorpha	Boule2	Not available
<i>Homo sapiens</i>		Boule	NP_001271291.1
<i>Hydra vulgaris</i>	Cnidaria	Boule1	JN379588
<i>Hydra vulgaris</i>	Cnidaria	Boule2	JN379589
<i>Hydra vulgaris</i>	Cnidaria	Boule3	JN379590
<i>Macrostomum lignano</i>	Flatworm (Turbulera)	Boule1	HM222645
<i>Macrostomum lignano</i>	Flatworm (Turbulera)	Boule2	JF911416
<i>Macrostomum lignano</i>	Flatworm (Turbulera)	Boule3	JF911417
<i>Mus musculus</i>	Mouse	Boule	NM_029267.3
<i>Nematostella vectensis</i>	Cnidaria	Boule1	XM_001635170
<i>Nematostella vectensis</i>	Cnidaria	Boule2	XM_001637198
<i>Saccoglossus kowalevskii</i>	Hemichordate	Boule	XM_011683988.1
<i>Schistosoma mansoni</i>	Flatworm (Trematode)	Boule	XM_002575473
<i>Schmidtea mediterranea</i>	Flatworm (Turbulera)	Boule1	KU519616
<i>Schmidtea mediterranea</i>	Flatworm (Turbulera)	Boule2	KU519617
<i>Strongylocentrotus purpuratus</i>	Echinoderm	Boule	XM_011683988.1

<i>Tribolium castaneum</i>	Arthropod (Coleoptera)	Boule	EFA05679
<i>Lingula anatina</i>	Brachiopod	Boule	http://marinegenomics.oist.jp/
<i>Pinctada fucata</i>	Pearl Oyster	Boule	http://marinegenomics.oist.jp/
<i>Biomphalaria glabrata</i>	Snail	Boule	XR_001216766.1
<i>Lottia gigantea</i>	Sea snail	Boule	http://genome.jgi.doe.gov/
<i>Capitella sp. I ESC-2004</i>	Polychaete	Boule1	http://genome.jgi.doe.gov/
<i>Capitella sp. I ESC-2004</i>	Polychaete	Boule2	http://genome.jgi.doe.gov/
<i>Octopus bimaculoides</i>		Boule	XM_014929311.1
<i>Helobdella robusta</i>	Leech	Boule1	http://genome.jgi.doe.gov/
<i>Helobdella robusta</i>	Leech	Boule2	http://genome.jgi.doe.gov/
<i>Echinococcus multilocularis</i>	Flatworm (Cestode)	Boule1	parasite.wormbase.org
<i>Echinococcus multilocularis</i>	Flatworm (Cestode)	Boule2	parasite.wormbase.org
<i>Hymenolepis diminuta</i>	Flatworm (Cestode)	Boule1	parasite.wormbase.org
<i>Hymenolepis diminuta</i>	Flatworm (Cestode)	Boule2	parasite.wormbase.org
<i>Hymenolepis microstoma</i>	Flatworm (Cestode)	Boule	parasite.wormbase.org
<i>Taenia asiatica</i>	Flatworm (Cestode)	Boule	parasite.wormbase.org
<i>Danio rerio</i>	Zebrafish	Dazl	AB018191.1
<i>Gallus gallus</i>	Aves	Dazl	NM_204218.1
<i>Mus musculus</i>	Mouse	Dazl	NM_010021.5
<i>Homo sapiens</i>		DAZL	NM_001190811.1
<i>Oryzias latipes</i>	Japanese killfish	Dazl	NP_001098269.1
<i>Xenopus laevis</i>	Frog	Dazl	AF017778.1
<i>Homo sapiens</i>		DAZ	U21663.1

Table S4. Cloning and qRT-PCR primer sequences**Cloning primers**

Gene name	Forward cloning primer	Reverse cloning primer	Genbank accession number
<i>boule1</i>	TGCAAACAAAATGTCAACTGAT	CATAAGGCACGGGTCCAT	KU519616
<i>boule2</i>	TATTTGTTGGGGGATTTC	CTTTGAGGTGTTGCCATTGA	KU519617
<i>CDC25-1</i>	TCACAACACTCCTGAAACACCA	TTCTGGTCCACGAACCGATG	KU852687
<i>CDC25-2</i>	ATGCAATATTTCTGTCAGTC	AAGACGCTTAATATCACATC	KU852688
<i>CDC25-3</i>	TGGCCACCTGTTTATTCCTC	CGACTTGACAATTCCCATCA	KU852689
<i>DAZAP1</i>	GATGGTAACGAGATTGGAAA	TATGACGTTGTTTGGTTTGA	KU852669
<i>DAZAP2</i>	TGACGGTGTCTATAAAAGTCA	AGCTCCTTGATCCCATAAAT	KU852670
<i>DZIP/iguana</i>	ATCACCGTTGTCCATATTGT	GTCATCCTCCAAATTTTCA	KU852671
<i>GRSF1-1</i>	TACAGGGGAGGCATTTGTTC	TCCGTCTGGGCCTATTTGTA	KU852676
<i>GRSF1-2</i>	GAGAAAGGCCACGAAGAGAA	AAAACATCATCTGGGCGTGT	KU852677
<i>PAM</i>	ACCGTCAGACCAAAACGAAC	TGCTTGAGCCACATCTGAAC	KU852680
<i>pumilio</i>	GGCAGGATTGTCTGAAGTCTCAG	CCAAGATCCTGATTGTTTTTCA	KU852681
<i>Ringo/SPY</i>	GCTCACGATGTCTGAAGAAGA	ATCTGACTCGTCGCTGTCAT	KU852682
<i>SDAD1</i>	TGGAGTTGCTGTCGAGATTG	CTTTCGGTTTTTGCTTGCTC	KU852686
<i>TPX1</i>	TTCTAACCGCCCATAACACC	CAGAGTCCGTCATTGCATGT	KU852672
<i>TRF2-1</i>	CACTTTTCCAGTGGTCATGATT	CATTGGACGCGAGTTCATAA	KU852673
<i>TRF2-2</i>	TTGTGATGCCTACACCTCAGTT	TTTTCCCGAAACGAAAATCA	KU852674
<i>TRF2-3</i>	TATCGCCTGCTTTTTCGACT	TCTTTTCTCCGGTCAAAAT	KU852675
<i>TSSK</i>	TTGCTGGAAATCGAGAACAG	ACTCGTCAGACTCGTTGCAC	KU852683
<i>vasa1</i>	TTGACCCCAGTGCAAAAATA	GCCAACAACCTCAACAGCTA	KU852684
<i>vasa2</i>	TTCCAACGCGTGAATTATGT	GTCGCCATGGATTGTAGTTG	KU852685

qRT-PCR primers

Gene name	Forward qRT-PCR primer	Reverse qRT-PCR primer
<i>boule1</i>	TCAAGCAACGATGACTGCTG	TTTGACGTGATTCCACCAAC
<i>boule2</i>	CCACTATCAATGGCAACACC	TCAACGGTTCTACTGGCATC
<i>nanos</i>	CAAGGACAAATGTTGCCTGTA	CAACCCATCGATCCAACCTCT
<i>pka</i>	CATAGTCCAAGGCGATGATG	GGCGTTGTACATCAGTGCTAGT
<i>tkn-1</i>	CTGACATGCTTGGCACTTCT	GCGGTCTTCCCTATTCACTT
<i>β-tubulin</i>	TGGCTGCTTGTGATCCAAGA	AAATTGCCGCAACAGTCAAATA