A premeiotic function for boule in the planarian Schmidtea mediterranea

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Mutations in Deleted in Azoosperma (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 planar homologs of human DAZL/DAZ-interacting partners and DAZ family mRNA targets. Finally, our phylogenetic analyses indicate that premeiotic functions of planar 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.

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uman male infertility is often associated with Y chromosome microdeletion (1). In 1976, Tiepolo and Zuffardi proposed the existence of an azoosperma 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 Azoosperma (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\textsubscript{\textsubscript{5}}-ligated 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 Azoosperma (DAZ) family of RNA-binding proteins, consisting of Boule, Dazl-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 planar 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), KU852690 (DAZAP1), KU852670 (DAZAP2), KU852671 (DZP), KU852672 (GRSF1-1), KU852673 (GRSF1-2), KU852680 (PAM), KU852681 (Pumilio), KU852682 (RingoSP), KU852686 (SDAD1), KU852672 (TPX1), KU852673 (TRF2-1), KU852674 (TRF2-2), KU852675 (TRF2-3), KU852683 (TSTK), 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 Dazl/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 RRRMs.
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 μm.) (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 μm.)
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 spermatogonal 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.
(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 knockdowns 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
additional control, we performed a parallel experiment with *dmd1* 
(SI Appendix, Fig. S6A), a gene previously shown to be required 
for SSC respecification (29). We confirmed gene knockdowns at 
14 d postamputation by quantitative real time-PCR (qRT-PCR) 
(SI Appendix, Fig. S6B).

To test whether *boule1* or *boule2* is required for the 
maintenance and differentiation of early germ cells post-
specification, we performed gene knockdowns on sexual 
hatchlings (<48 h posthatching). At this stage of develop-
ment, the male gonad of sexual planarians consists of small 
clusters of *nanos*+ SSCs and *dmd1*+ somatic gonadal cells, 
enabling us to examine the consequences of *boule1* or *boule2* 
loss on early male germ cells in the absence of more differ-
entiated cells. When control animals reached adulthood after 
~12 feedings of dsRNA, they exhibited robust spermatogenesis 
in all samples (*n* = 14/14, Fig. 2C). SSCs in *boule1*(RNAi) an-
imals are able to progress through mitosis and form clusters of 
spermatogonia, but are unable to produce meiotic and post-
meiotic cells (*n* = 13/13, Fig. 2C). *boule2*(RNAi) animals com-
pletely lack male germ cells (*n* = 14/14, Fig. 2C). We also 
imaged the hatchlings after two and four feedings of dsRNA to 
further confirm that the two genes are required for early germ 
cell maintenance. We found that the knockdown phenotypes 
are similar to the phenotype seen in sexually mature adults (SI 
Appendix, Fig. S6 C and D). Experiments on animals regener-
ating their reproductive system (29) also showed comparable 
results (SI Appendix, Fig. S7).

Together, these experiments show that neither *boule1* nor 
*boule2* is necessary for the specification of male germ cells; how-
ever, the two genes perform distinct roles in male germ cells 
after they are specified. *boule1* is required for meiotic progression, and 
*boule2* is required for the maintenance of the earliest male germ 
cells, *nanos*+ SSCs. Our observation that *boule2* is not necessary 
for the specification of SSCs, but is required for the maintenance 
and differentiation of early male germ cells, is similar to the *Dazl* 
null phenotype seen in vertebrates (12, 13, 17), further lending 
support to the hypothesis that planarian *boule2* and vertebrate 
*Dazl* perform similar functions.

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**Fig. 5.** Knockdown of putative DAZ family targets phenocopies *boule2*(RNAi). (A) *SDAD1*, *CDC25-1*, and *CDC25-2* are enriched in planarian testes. (Scale bars, 1 mm.) (B–E) Animals fed control, *SDAD1*, *CDC25-1*, and *CDC25-2* dsRNA (three feedings spaced 4–5 d apart) labeled with *gH4* and *tkn-1*. Similar to *boule2*(RNAi), RNAi knockdown of these putative targets results in animals having fewer spermatogonia, and the spermatocyte layer remains intact. Continued *SDAD1*(RNAi) and *CDC25-1*(RNAi) results in lysis, whereas (F) *CDC25-2*(RNAi) animals lose all male germ cells over time. (Scale bars, 50 μm.)
**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, whereas early gH4+ female germ cells were still present (n = 4/4 for all samples, Fig. 3C). The dual role of planarian boule genes in both testses 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 Expresssed 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 (Methods). 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—DAZAPI, 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). DAZAPI(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 DAZAPI knockout mice, which lack mature male gametes (35), DAZAPI 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.](image-url)
**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** regulates either lacked testes (n = 2/6) or had regressed testes 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 planar **DZIP** gene, known as **Smed-iguana**, has previously been shown to be required for ciliogenesis in asexually planarians (36). Regenerating **iguana**(RNAi) axenals are able to produce normal blastemas, but do not form ciliated epidermis (leading to defects in cilia-driven locomotion) or ciliated proto-nephridia (resulting in bloating and blistering defects due to disrupted osmoregulatory function) (36). **iguana**(RNAi) in the sexual strain led to budding 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**(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 meiotic 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 (PABPC), (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 **<i>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. **SDADI**, a homolog of the yeast gene *severe depolymerization of actin*, is a putative target of human DAZL and PUMILIO 2 (44). A function for **SDADI** in spermatogenesis has not been reported previously. By ISH, we find that **Smed-SDADI** was detected in the testes as well as soma (Fig. 5A). RNAi experiments showed that **SDADI** 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). **SDADI**(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 **SDADI** 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. S9 D 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) regeneration—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. S9 F 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 Daz1, 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 Boule 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 Macrostreamum 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 function, suggesting 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 Macrostreamum lignano. This result is consistent with neofunctionalization of planarian and vertebrate premeiotic Boule derivatives.

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 pcJS3.2 (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 blast 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 required protein blast of which to NCBI protein databases did not yield DAZAP1 as the highest hit. No sequences corresponding to DAZAP2 and DAZIP1/2/3 were found. Cloning primers are in SI Appendix, Table S4.

dRNA Synthesis and RNAi. cDNAs corresponding to boule1 and boule2 cloned in pcJS3.2 (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 (RTP, ATP, and TTP final 10 mM each), 1.7 μL thermostable inorganic phosphoxygenase (TIPP; 2000 units/ml; New England Biolabs), 0.5 μL recombinant ribonuclease inhibitor (RNasin; 2500 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 3:1:1 liver:Montjuïc salts mix. Control animals were fed dsRNA synthesized from a nonplanarian gene inserted in pcJS3.2.

Riboprobe Synthesis. boule1 and boule2 cDNA cloned in pcJS3.2 (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 1066 mM rNTPs (RTP, ATP, and TTP final 10 mM each), UTP 6 mM final (Promega), 0.4 μL of Digoxigenin-12-UTP (Roche), 0.6 μL recombinant ribonuclease inhibitor (RNasin, 2500 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 RMRMs (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 500 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. 0.11 (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% H2O2) 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/ul. 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 MgCl2, 0.1% Tween-20 brought up to volume with 10% polyvinylalcohol (Sigma)) containing 4.5 µl/ml NBT and 3.5 µl/ml BCIP (Roche). For FISH, development was done in freshly made Tyramide solution (Fluor-tyramide (1:250-1:500), 4-IPBA (1:1000), and H2O2 (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 CoCl₂, 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 µg/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 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 ΔΔCT. 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 µm.

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 µm. 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 µm. (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 µm.

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 µm.

**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 µm.

**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 µm.
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 µm. (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*<sup>+</sup> and *gH4*<sup>+</sup> 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 µm.

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 RRMss. (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

**boule1 mRNA**

Dorsal (Testes)

Ventral (Ovaries)

**boule2 mRNA**

Dorsal (Testes)

Ventral (Ovaries)
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Figure S2
Figure S3

A

Neoblast → SSCs → Spermatogonia → Spermatocytes → Spermatids → Spermatids → Spermatozoa

- Control (RNAi)
- boule1(RNAi)
- boule2(RNAi)

B

Early knockdown
- SSCs (nanos)
- Spermatids (pka)
- DNA (DAPI)

C

Late knockdown
- SSCs (nanos)
- Spermatids (pka)
- DNA (DAPI)
Figure S4

A

Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
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Smed-boule1
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Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG
Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG
Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG
Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG
Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG
Smed-boule1
CTGCTGCGAAACCCTATAGCATG
Smed-boule2
CTGCTGCGAAACCCTATAGCATG

B

C

D

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

Control (RNAi)  boule1(mRNA)  boule2(mRNA)
Figure S5

A

Number of TUNEL+ cells per testis lobe

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

B

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

TUNEL  DAPI

TUNEL  DAPI

TUNEL  DAPI
Figure S6

A

nanos/DAPI

nanos

B

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<tr>
<th></th>
<th>Control (RNAi)</th>
<th>boule1 (RNAi)</th>
<th>Control (RNAi)</th>
<th>boule2 (RNAi)</th>
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<tr>
<td>Relative mRNA levels</td>
<td>1.2 ± 0.1</td>
<td>* 0.8 ± 0.1</td>
<td>** 1.4 ± 0.1</td>
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- ** boule1 mRNA
- ** boule2 mRNA

C

nanos/DAPI  gH4/DAPI

D

nanos/DAPI  gH4/DAPI

Control (RNAi)  boule1(RNAi)  boule2(RNAi)
Figure S7

A

Amputate

7 days post amputation

Sexually immature planarians

qRT-PCR to confirm absence of differentiated cells

Start dsRNA feedings

B

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

nanos DAPI

gH4 DAPI

C

Relative mRNA levels

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<th>Amputated</th>
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<td>pka</td>
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***  ****
Figure S8

**A** Control (RNAi) - homeostasis

**B** DAZAP2(RNAi) - homeostasis

**C** DAZAP1(RNAi) - homeostasis

**D** iguana(RNAi) - homeostasis

**E** Control (RNAi) - regenerates

**F** DAZAP1(RNAi) - regenerates
Putative DAZL/DAZ targets are expressed in the male germline of *S. mediterranea*.

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

- **B** Control (RNAi)
- **C** *SDAD1*(RNAi)
- **D** *CDC25-1*(RNAi)
- **E** *CDC25-2*(RNAi)

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

- **F** Control (RNAi) - regenerates
- **G** *CDC25-2*(RNAi) - regenerates
Figure S10

A

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<th>Organism</th>
<th>Protein</th>
<th>Amino Acid Sequence</th>
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<td>S. cerevisiae HRP1</td>
<td>KADLSKESCR MFICGLNND-</td>
<td>TIDNLREYE CYK--GTV DLKIMDPD-</td>
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<td>H. vulgaris Boule2</td>
<td>VIYTHYPKR LFVICPPDP-</td>
<td>AGAEQFLFFE ANY--GTVV EAVVTVL-</td>
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<td>N. vectensis Boule1</td>
<td>FLLKPRYPKR IFVGGPDP-</td>
<td>TIIAELAFF ADE--GTVV ESKVFDIPD-</td>
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<td>H. miamia Boule1</td>
<td>SKSHLPRIK IFVGGPDP-</td>
<td>TIDNLREYF AEN--GTVV ESKVFDIPD-</td>
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<td>H. vulgaris Boule3</td>
<td>SPGDLPRIPKR IFVGGPDP-</td>
<td>TIDNLREYF ETV--GTVV ESKVFDIPD-</td>
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<tr>
<td>N. vectensis Boule2</td>
<td>DPYLPRIPKR IFVGGPDP-</td>
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<td>M. lignano Boule1</td>
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<td>AEN--GTVV ESKVFDIPD-</td>
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<td>GENE----GAVR DVKIIADK-G</td>
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<td>M. musculus Dazl</td>
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<td>H. sapiens DAZL</td>
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<td>C. elegans Boule(Dazl)</td>
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<td>TEEELAFF AEN--GTVV ESKVFDIPD-</td>
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<td>C. briggsae Boule</td>
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<td>H. robusta Boule2</td>
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<td>P. fucata Boule</td>
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<td>D. ananassae Boule</td>
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<td>GENE----GAVR DVKIIADK-G</td>
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<td>S. kowalevskii Boule</td>
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</table>

B

Branch length ratio

Macrostomum
Schmidtea
Gallus
Mus
Homo

Branch length ratio

0 10 20 30 40 50
Table S1. Experimental details for planarian homologs of DAZ-associated proteins

<table>
<thead>
<tr>
<th>Associated protein name</th>
<th>Required for regeneration</th>
<th>Expressed in male germ cells?</th>
<th>Germ cell RNAi phenotype in adult sexually mature worms</th>
<th>Germ cell RNAi phenotype in hatchlings</th>
<th>Required for de novo specification of germ cells?</th>
<th>RNAi phenotype in sexually immature regenerates</th>
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<tbody>
<tr>
<td>DAZAP1</td>
<td>No</td>
<td>Yes</td>
<td>No mature sperm</td>
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<td>DAZAP2</td>
<td>No</td>
<td>Yes</td>
<td>No phenotype</td>
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<td>DAZAP1+2</td>
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<td>No mature sperm</td>
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<td>Not tested</td>
<td>Not tested</td>
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<td>DZIP/Smed-iguana</td>
<td>Yes (bloating observed in homeostasis)</td>
<td>Yes</td>
<td>No mature sperm</td>
<td>Not tested</td>
<td>Lysis</td>
<td>Lysis, no testes in remaining fragments</td>
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<td>pumilio</td>
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<td>Yes</td>
<td>Small testes, no loss of specific cell type marker</td>
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Table S2. Experimental details for planarian homologs of DAZ family targets

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<th>Expressed in the male germ cells?</th>
<th>Germ cell RNAi phenotype in adult sexually mature worms</th>
<th>Germ cell RNAi phenotype in hatchlings</th>
<th>Required for de novo germ cell specification?</th>
<th>RNAi phenotype in sexually immature regenerates</th>
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<td>CDC25-1</td>
<td>Yes</td>
<td>Yes</td>
<td>Loss of early germ cells followed by more differentiated cells</td>
<td>No male germ cells</td>
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<td>CDC25-2</td>
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<td>Loss of early germ cells followed by more differentiated cells</td>
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<td>vasa1</td>
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<td>vasa2</td>
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<td>Ringo/SPY</td>
<td>No</td>
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<td>Small testes with only SSCs and spermatogonia</td>
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<td>Boule</td>
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<td>Pearl Oyster</td>
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<td>Boule</td>
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<td><strong>Lottia gigantea</strong></td>
<td>Sea snail</td>
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<td><strong>Capitella sp. I ESC-2004</strong></td>
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