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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 *boule*2 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 *daz1* 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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The authors declare no conflict of interest.

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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 *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 RRMs

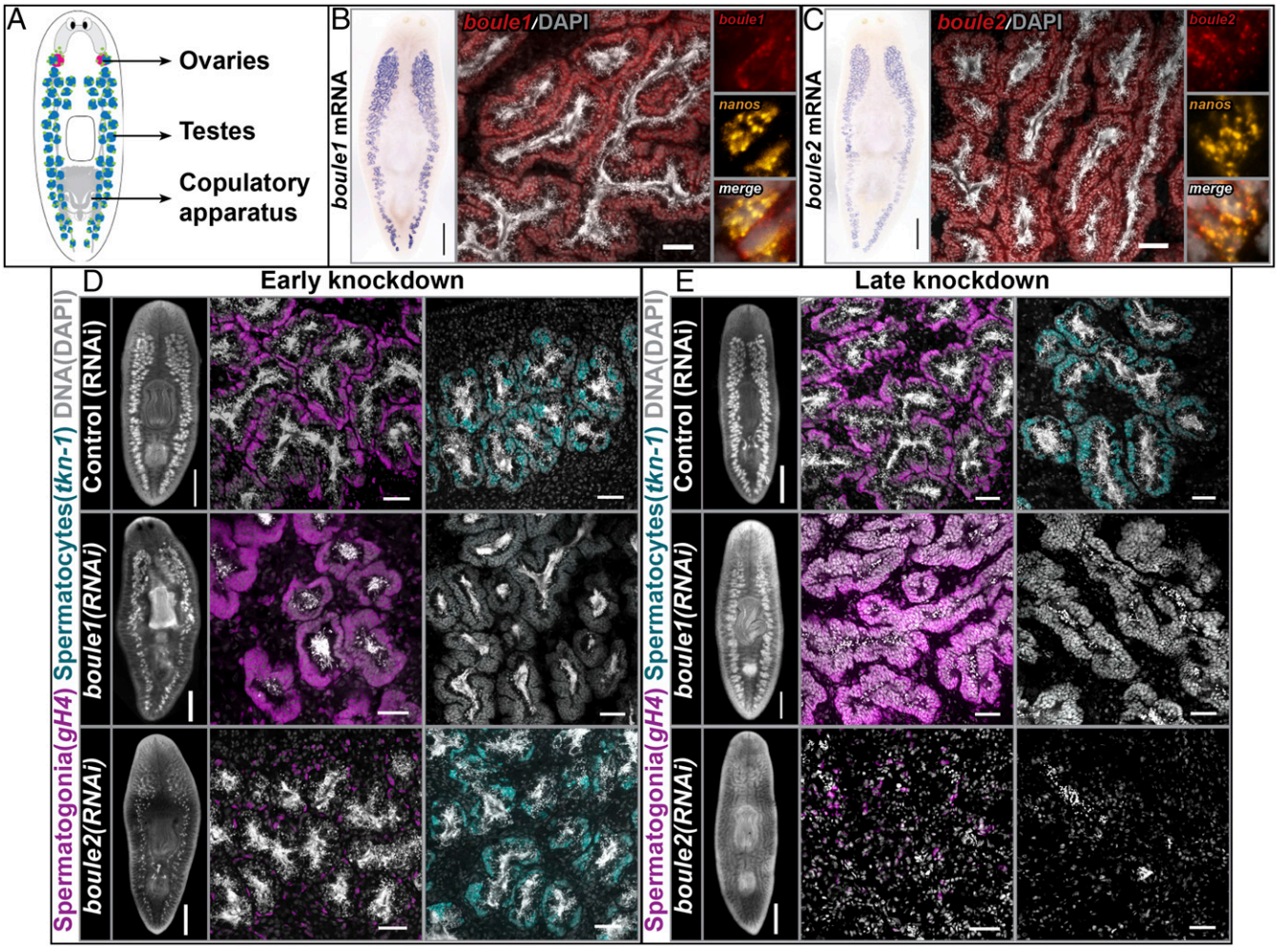

Fig. 1. Planarian *boule1* and *boule2* perform different functions in spermatogenesis. (*A*) Illustration of sexual planarian depicting the positions of reproductive structures. Ovaries are in red, testes are in blue, and germ-line stem cells are in green. (*B* and *C*) Colorimetric ISH showing *boule1* 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 μm.) 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 μm.)

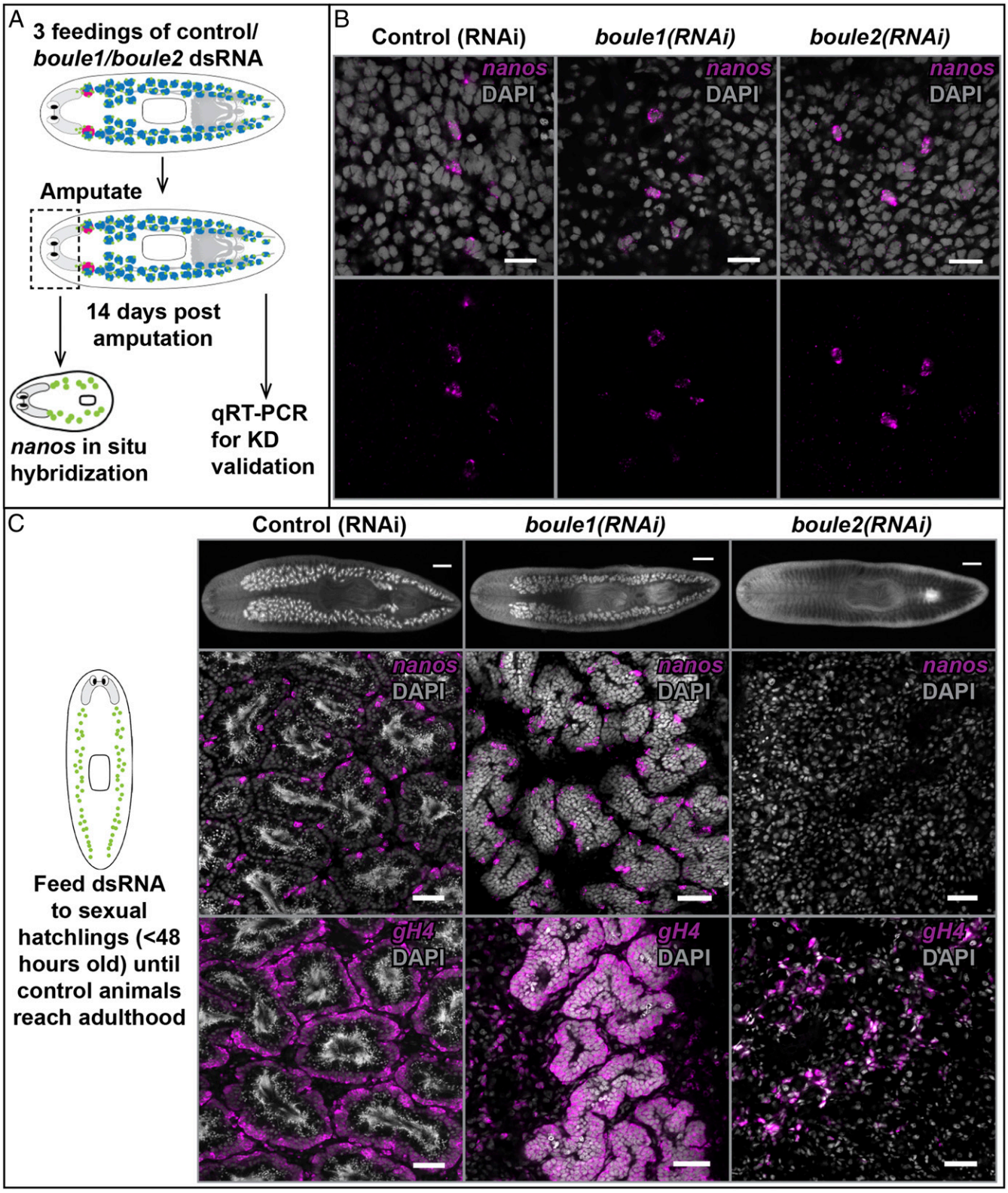

Fig. 2. *boule2* is required for maintenance of early male germ cells but not required for respecification of SSCs. (*A*) Experimental scheme for testing the requirement of a gene for de novo respecification of SSCs. Animals are fed control/*boule1*/*boule2* dsRNA three times and amputated anterior to 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. 1*A*). Both *boule1* and *boule2* were expressed in male and female gonads (Fig. 1 *B* 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. 1 *B* 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. S3*A*) (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. S3*A*) (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. 1*D*). This spermatocyte loss was accompanied by a concomitant increase in the *germinal histone H4*$^+$ (*gH4*$^+$) mitotic spermatogonial layer (23, 24) (n = 6/6, Fig. 1*D*). At this RNAi timepoint, *boule1(RNAi)* animals showed no discernible changes in the *nanos*$^+$ SSC population (*SI Appendix*, Fig. S3*B*). 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. S3*B*). 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. 1*E* and *SI Appendix*, Fig. S3*C*).

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

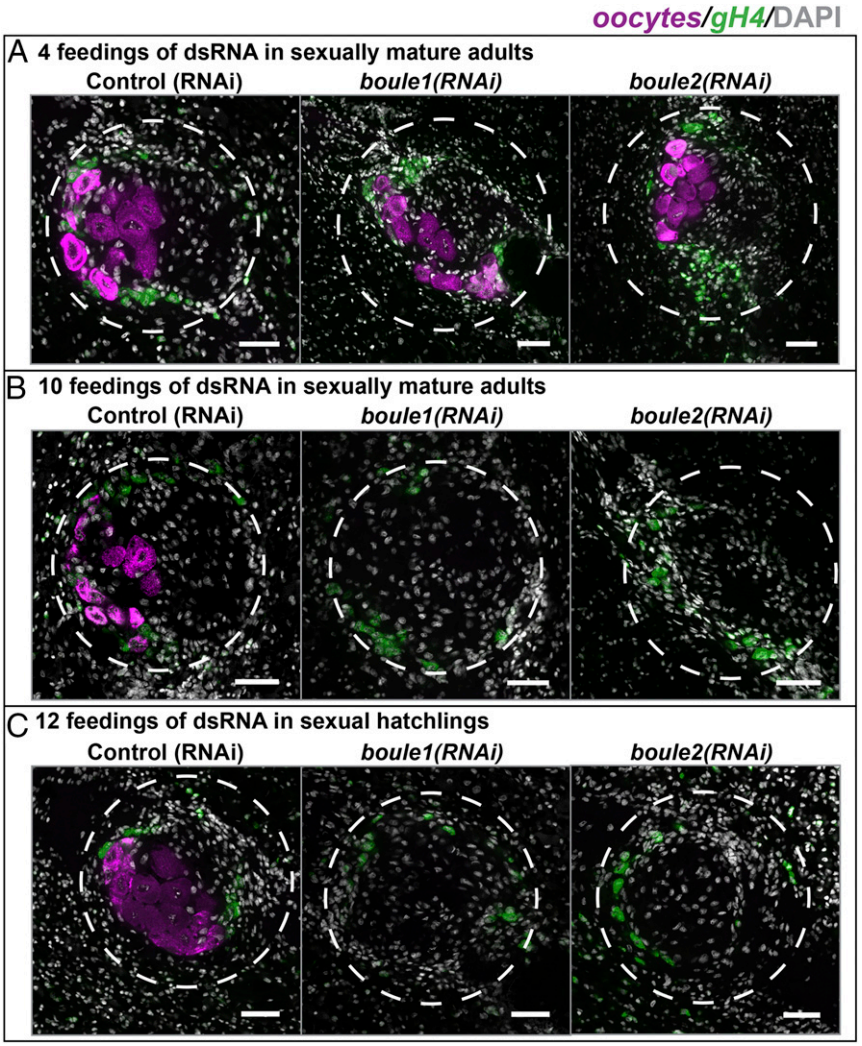

Fig. 3. Planarian *boule* genes play a role in the ovaries. (*A*) Following 4 feedings of dsRNA, *boule1(RNAi)* and *boule2(RNAi)* female gonads appear similar to controls. Oocytes are marked using *Contig2621* (magenta) (26), and *gH4* (green) labels early female germ cells. (*B*) Following 10 feedings 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. 1*D*). Half of *boule2(RNAi)* animals (n = 3/6) had no *nanos*$^+$ SSCs (*SI Appendix*, Fig. S3*B*); *pka*$^+$ spermatids appeared unaffected in *boule2(RNAi)* animals at these early stages (*SI Appendix*, Fig. S3*B*). 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. 1*E* and *SI Appendix*, Fig. S3*C*).

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. 2*A*) (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. 2*B*). As an

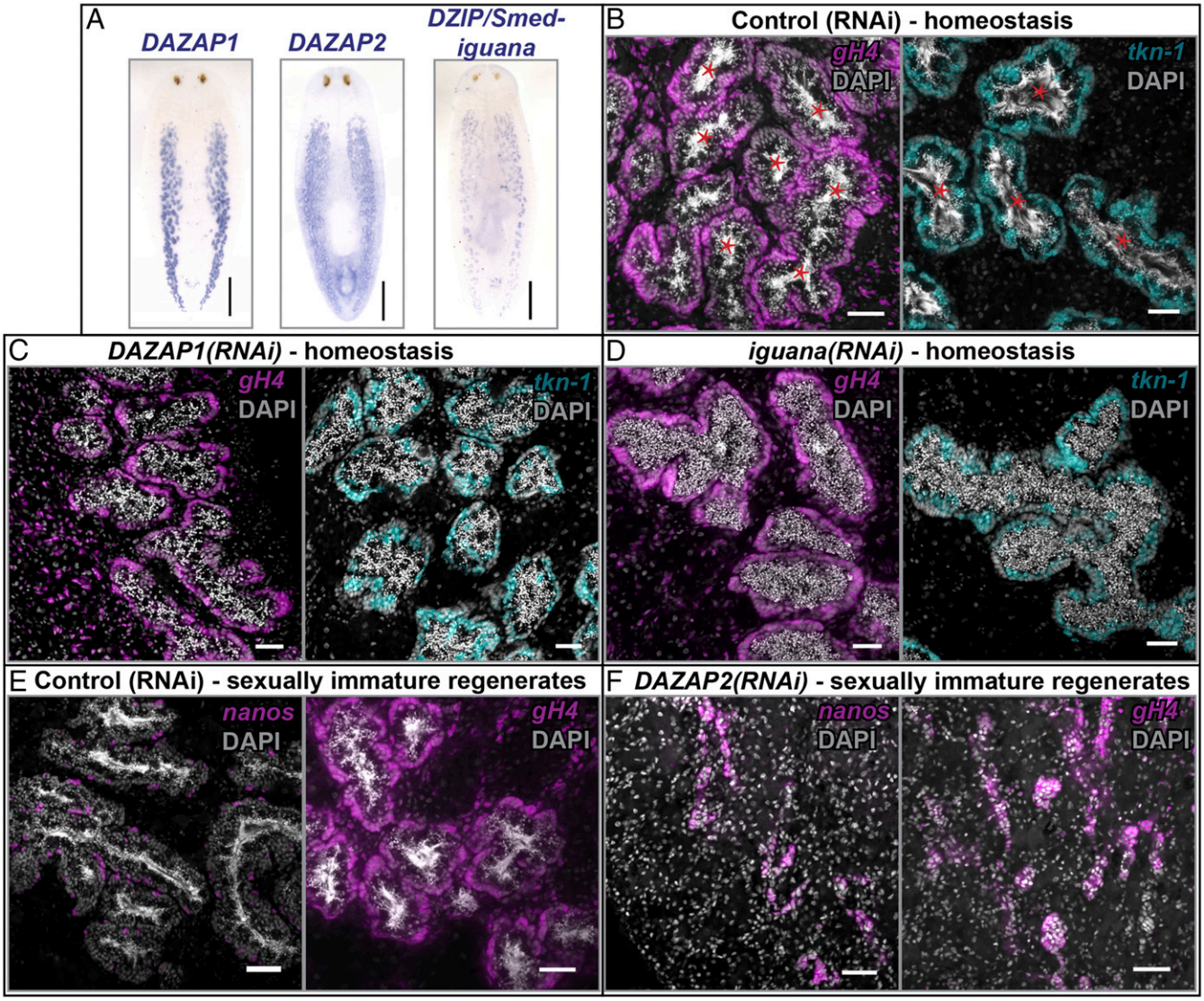

Fig. 4. Homologs of vertebrate DAZ-associated proteins are expressed and function in the testes of *S. mediterranea*. (*A*) *DAZAP1*, *DAZAP2*, and *DZIP (Smed-iguana)* transcripts are detected in the testes by ISH. (Scale bars, 1 mm.) (*B*) Adults fed control dsRNA in homeostasis show robust spermiogenesis. 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 μm.)

additional control, we performed a parallel experiment with *dmd1* (*SI Appendix*, Fig. S6*A*), 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. S6*B*).

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 development, 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 differentiated cells. When control animals reached adulthood after ~12 feedings of dsRNA, they exhibited robust spermatogenesis in all samples (n = 14/14, Fig. 2*C*). SSCs in *boule1(RNAi)* animals 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. 2*C*). *boule2(RNAi)* animals completely lack male germ cells (n = 14/14, Fig. 2*C*). 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 regenerating 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; however, 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.

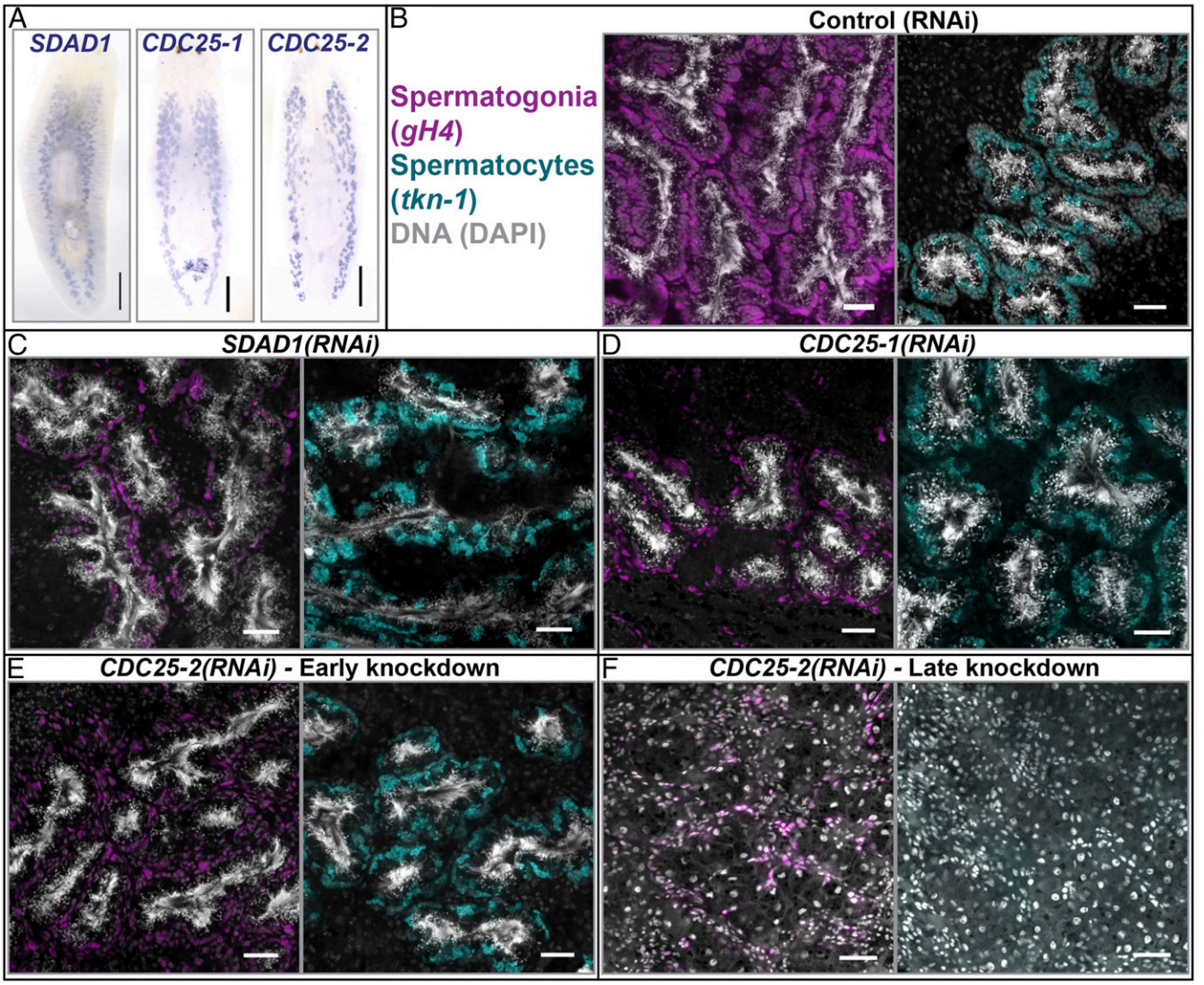

Fig. 5. Knockdown of putative DAZ family targets phenocopies *boule2(RNAi)*. (*A*) *SDAD1*, *CDC25-1*, and *CDC25-2* are enriched in planarian testes. (Scale bars, 1 mm.) (*B–E*) Animals fed control, *SDAD1*, *CDC25-1*, and *CDC25-2* dsRNA (three feedings spaced 4–5 d apart) labeled with *gH4* and *tkn-1*. 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. 3*A*). 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. 3*B*). 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. 3*C*). 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* (*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—*DAZAP1*, *DAZAP2*, and *DZIP* (*Smed-iguana*)—and found that these genes were expressed in the testes (Fig. 4*A*). 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. 4*C* 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.

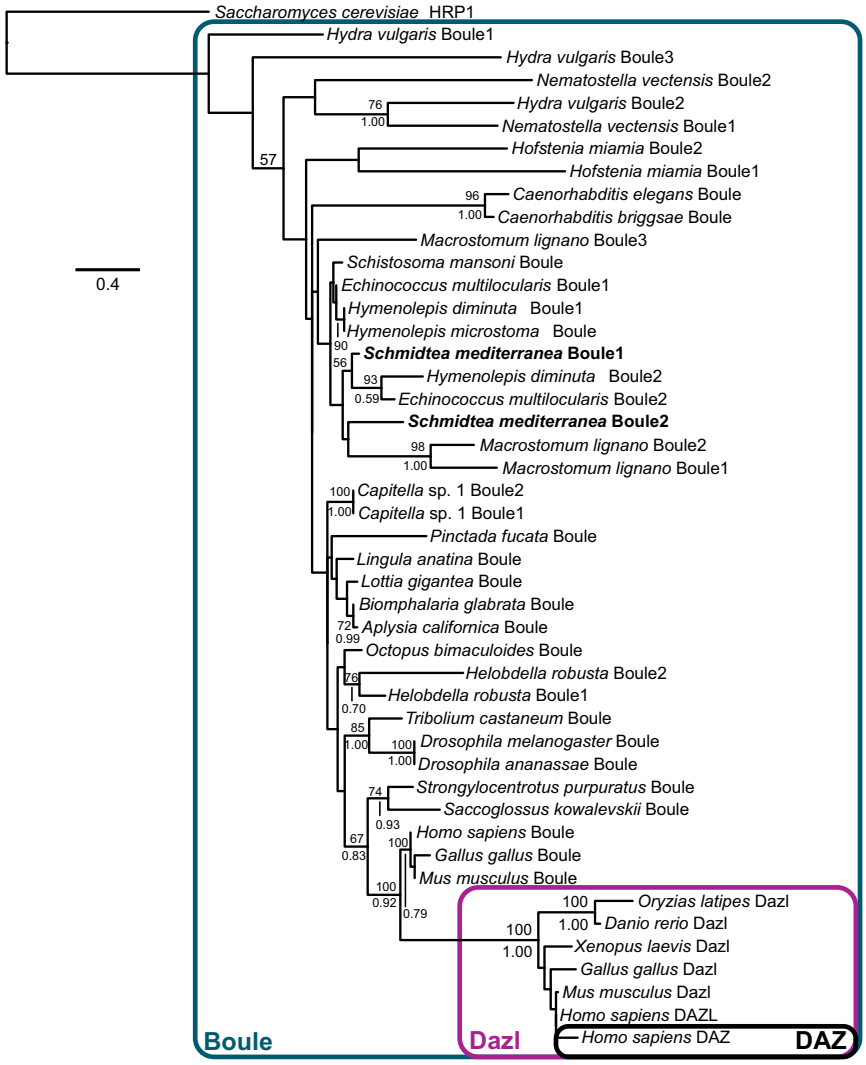

Fig. 6. Phylogenetic analysis reveals independent origins of planarian Boule2 and vertebrate Dazl. Phylogenetic tree topology of DAZ gene family from ML and BI analysis. Numbers above nodes indicate ML bootstrap resampling frequencies (500 replicates). Numbers below nodes indicate Bayesian posterior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

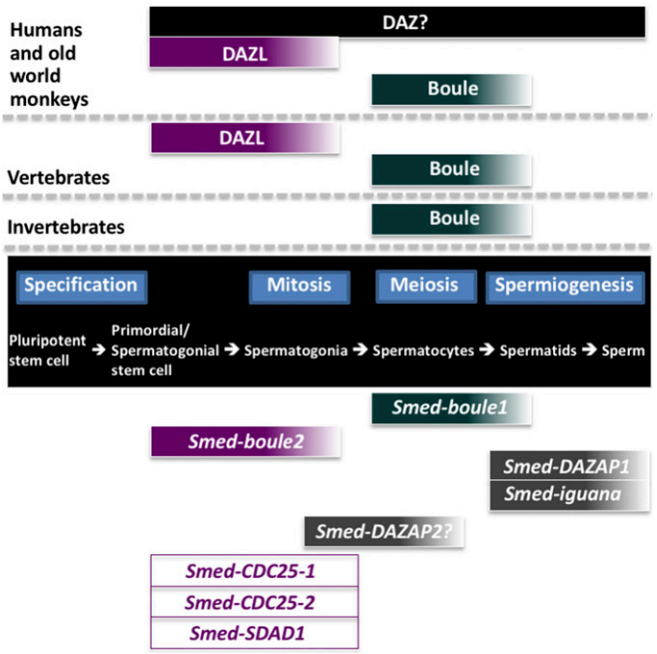

Fig. 7. Summary of DAZ family functions in planarians and other systems. (*Center*) Different stages of spermatogenesis. (*Upper*) Known functions of the DAZ family in male germ cell development in other systems. (*Lower*) Summary of the functions of planarian *boule* genes, their putative-associated 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. 4*D* and *SI Appendix*, Fig. S8*D*). 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. 5*A* and *SI Appendix*, Fig. S9*A* 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. 5*A*). RNAi experiments showed that *SDAD1* is required for maintenance of SSCs (n = 3/6) and spermatogonia (n = 6/6), similar to *boule2(RNAi)* (Fig. 5*C* and *SI Appendix*, Fig. S9*C*). *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. 5*A*). 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. 5 *D* 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. 5*F* 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. 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 *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. S10*A*), 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. S10*B*). 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 *DZIP/iguana* 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 $MgCl_2$, 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 denaturating 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 $MgCl_2$, 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 RRMs (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. S10*A*. 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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