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(Article begins on next page)
A premeiotic function for boule in the planarian Schmidtea mediterranea

Harini Iyer,a,b Melanie Issigonis,a,b Prashant P. Sharma,c,1 Cassandra G. Extavour,5 and Phillip A. Newmark,a,b,2

Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801; Howard Hughes Medical Institute, University of Illinois at Urbana-Champaign, Urbana, IL 61801; and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138

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

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 planar- nian 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. KUS519616 (boule1), KUS519617 (boule2), KUS52687 (CDC25-1), KUS52688 (CDC25-2), KUS52689 (CDC25-3), KUS52690 (DAAZP1), KUS52670 (DAAZP2), KUS52671 (DZP), KUS52672 (GRSF1-1), KUS52673 (GRSF1-2), KUS52680 (PAM), KUS52681 (Pumilio), KUS52682 (RingoSP), KUS52686 (SADAI1), KUS52672 (TRF1X), KUS52673 (TRF1-2), KUS52674 (TRF2-2), KUS52675 (TRF2-3), KUS52683 (TSSK), KUS52684 (Vasa1), and KUS52685 (Vasa2)].

aPresent address: Department of Zoology, University of Wisconsin-Madison, Madison, WI 53706.
b1To whom correspondence should be addressed. Email: pnewmark@life.illinois.edu.

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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.](https://www.pnas.org/doi/10.1073/pnas.1521341113)

(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. \textit{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/\textit{boule1}/\textit{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 \textit{nanos} FISH and RNA is extracted from the tail fragment to ensure that test mRNA levels are reduced. \textit{nanos} labels planarian SSCs. (B) Control (RNAi), \textit{boule1}(RNAi), and \textit{boule2}(RNAi) animals all show respecification of \textit{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. \textit{boule1}(RNAi) animals have testis lobes with only SSCs (\textit{nanos}+) and spermatogonia (\textit{gH4}+). \textit{boule2}(RNAi) animals lack male germ cells; remnant \textit{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.

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

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

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 specifying 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 example, the number of nanos+ SSCs was similar to control animals. This was consistent with the observation that both boule1 and boule2 were dispensable for the maintenance of SSCs and spermatogonia, as shown in Figs. 1 and 2B.

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additional control, we performed a parallel experiment with dmd1 (SI Appendix, Fig. S6), 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-spezification, 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. 2C). 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. 2C). boule2(RNAi) animals completely 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 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.
boule1 and boule2 Are Necessary for Oogenesis. We examined the role of boule1 and boule2 in the ovaries by carrying out gene knockdowns for different lengths of time and during different developmental stages. Following 4 dsRNA feedings ( spaced 4–5 d apart), ovaries of boule1(RNAi) and boule2(RNAi) animals appeared comparable to controls (n = 6/6 for all samples, Fig. 3A). However, following prolonged gene knockdown (10 feedings over a period of ~2 mo), both boule1(RNAi) (n = 4/4) and boule2(RNAi) (n = 6/6) animals lacked oocytes, whereas early gH4+ female germ cells were still present (Fig. 3B). Similarly, when sexual hatchlings were fed boule1 and boule2 dsRNA over a period of 2 mo, the animals lacked mature oocytes, but gH4+ female germ cells were present (n = 4/4 for all samples, Fig. 3C). The dual role of planarian boule genes in both testes and ovaries is especially interesting because, with the exception of Dazl, other members of the DAZ family (boule orthologs in various systems and DAZ) appear restricted in function exclusively to the male or the female germ line.

Homologs of Vertebrate DAZ-Associated Proteins Are Expressed and Function in the Testes of *S. mediterranea*. Yeast two-hybrid screens and other in vitro studies (31–34) have identified several potential DAZL/DAZ-interacting partners using human DAZ as bait. Homologs of these genes have not been described in *C. elegans* and *D. melanogaster*, which only possess meiotic boule (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. 4A). To determine the role of these genes in spermatogenesis, we performed RNAi during homeostasis (in sexually mature adults), during development (in hatchlings), as well as in sexually immature regenerates (animals fed dsRNA three times, amputated prepharyngeally to induce regression of testes (29), and refed dsRNA during regeneration). DAZAP1(RNAi) animals lacked elongated spermatids and mature sperm, whereas other male germ cells appeared intact in all three experimental conditions (n = 6/6 for all; Fig. 4B 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.

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**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.
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 DAZAP2) is uncertain (40–44). We identified and cloned a number of planarian homologs of putative DAZ family targets (Fig. S4 and SI Appendix, Fig. S9A and Table S2), but we will focus on the putative targets with germ cell RNAi phenotypes.

SDAD1, a homolog of the yeast gene severe depolymerization of actin, is a putative target of human DAZL and PUMILO 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. S4A). SDAD1 transcripts are DAZ family targets (40–44). We identified and cloned a number of planarian homologs of putative DAZ family targets (Fig. S4 and SI Appendix, Fig. S9A and Table S2), but we will focus on the putative targets with germ cell RNAi phenotypes.

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The CDC25 homolog twin 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 (44)) and will not be discussed here), designated CDC25-1 and CDC25-2, were expressed in testes (Fig. S4A). 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. S5D and E). The numbers of SSCs and spermatids were largely unaffected at the initial stages of knockdown (SI Appendix, Fig. S9D and E); at later stages, all male germ cells were absent (Fig. 5F and SI Appendix, Table S2). We next tested the requirement of CDC25-1 and CDC25-2 for specification and maintenance of early germ cells in sexual regenerates. CDC25-1(RNAi) animals do not regenerate, and undergo lysis, but there are no male germ cells present in regenerates before lysis (SI Appendix, Table S2). CDC25-2(RNAi) animals undergo morphogenesis defects in early germ cells (SI Appendix, Figs. S9F and G). The in vitro prediction that these transcripts are DAZ family targets in other systems, combined with the similarity of RNAi phenotypes between these genes and boule2, makes these transcripts strong candidates for putative targets regulated by planarian Boule2.

Premeiotic Functions of the DAZ Family Evolved Independently in Planarians and Vertebrates. Vertebrate Dazl, which plays a premeiotic role in germ cells, arose either during vertebrate evolution, or was present in a last common bilaterian ancestor and was
subsequently lost in some invertebrates. Based on the presence of a single DAZ family representative, boule, in both C. elegans and D. melanogaster, phylogenetic analyses, and comparison of gene structure and intron/exon counts, it has been proposed that Dazl arose through duplication of boule in the vertebrate stem lineage (9). Our identification of multiple paralogs of boule in an invertebrate model system, combined with the premeiotic germ cell function for one of these paralogs, provides us valuable tools for testing this hypothesis in a phylogenetic context.

We obtained multiple Boule sequences from diverse animal phyla (accession nos. in SI Appendix, Table S3; alignments in SI Appendix, Fig. S10A), placing special focus on invertebrates with multiple annotated Boule homologs. We performed both maximum likelihood (ML) (46) and Bayesian inference (BI) (47) analyses and found that S. mediterranea Boule paralogs were recovered in a clade formed by other platyhelminth Boule orthologs (Fig. 6). The short patristic distance between S. mediterranea 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 divergence) of premeiotic and meiotic DAZ family members in two planarians and three vertebrates, with branch lengths drawn from the Bayesian postburnin tree set (SI Appendix, Fig. S10B). For both S. mediterranea and the vertebrates, the distributions of ratios of premeiotic paralog branch lengths to meiotic paralog branch lengths were highly comparable, in contrast to the ratio distribution for the Boule proteins of Macrostomum lignano. This result is consistent with neofunctionalization of planarian and vertebrate premeiotic Boule derivatives.

boule, Dazl, and DAZ play crucial and conserved roles in gametogenesis across the animal kingdom (Fig. 7). However, there is considerable phenotypic diversity caused by defects in the DAZ family 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 DAZAP1-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 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 tblastx 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 recombinant protein blast of which to NCBI protein databases did not yield DAZAP1 as the highest hit. No sequences corresponding to DAZAP2 and DAZIP4/gamma were found. Cloning primers are in SI Appendix, Table S4.

dsRNA 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 10x 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 (Roche), 1 μL 17 mM oligo, 1 μL thermostable inorganic pyrophosphatase (TIPP; 2,000 units/mL; New England Biolabs), 0.5 μL recombinant ribonuclease inhibitor (RNasin; 2,500 units/mL; Promega), and 0.5–2.5 μg of PCR product. Reactions were incubated at 37 °C overnight, then treated with 1 μL of RQ1 RNase-free DNase (Fisher Scientific) for 20 min at room temperature. Each reaction was brought up to 100 μL followed by denaturing and annealing at the following temperatures: 95 °C (3 min), 75 °C (3 min), 50 °C (3 min), and room temperature (5 min). dsRNA was precipitated using ammonium acetate (2.5 M final concentration) plus two volumes of 100% ethanol. dsRNA (0.4–1 μg) was mixed with 10 μL of 3:1 liver:Montjuïc salts mix. Control animals were fed dsRNA synthesized from a nonplanarian gene inserted in 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 10x high yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl2, 20 mM spermidine, 0.1 M DTT), 1 μL 106 mM rNTPs (Roche), and 10 μM final rUTP (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. HRPI 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 branch temperatures, were run for 2 × 108 generations, with sampling every 2,000th generation. A mixed+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 × 108 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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