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# The roles of the *DAZ* family in spermatogenesis
## More than just translation?

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The *DAZ* family of genes are important fertility factors in animals, including humans. The family consists of Y-linked *DAZ* and autosomal homologs *Boule* and *Dazl*. All three genes encode RNA-binding proteins that are nearly exclusively expressed in germ cells. The *DAZ* family is highly conserved, with ancestral *Boule* present in sea anemones through humans, *Dazl* conserved among vertebrates and *DAZ* present only in higher primates. Here we review studies on *DAZ* family genes from multiple organisms and summarize the common features of each *DAZ* gene and their roles during spermatogenesis in animals. DAZ family proteins are thought to activate the translation of RNA targets, but recent work has uncovered additional functions. *Boule*, *Dazl* and *DAZ* likely function through similar mechanisms, and we present known functions of the *DAZ* family in spermatogenesis, and discuss possible mechanisms in addition to translation activation.



## Introduction

Infertility affects approximately 10% of couples, with half of these cases attributable to the male partner.[1] Though many of these cases are idiopathic, a high proportion of men with non-obstructive azoospermia (no sperm produced) have a microdeletion on the Y chromosome.[2] The discovery of such deletions led to the proposal of an "Azoospermia Factor" (AZF) as a genetic cause for some cases of infertility.[2] The AZF region has been further mapped into the three candidate regions AZFa, AZFb and AZFc,[3,4] each deleted in subsets of infertile men. Among the handful of genes in these regions, *Deleted in Azoospermia* (*DAZ*) was found to be deleted in 12–15% of a cohort of azoospermic men, making it a strong candidate for the AZFc gene.[5] *DAZ* is part of a large gene family (*DAZ* family) with autosomal homologs *Dazl* (*DAZ-Like*) and *Boule*, all of which encode RNA-binding proteins.[6-9] *DAZ* family genes are reproduction-specific and present in nearly all animals,[10] making them an important gene family in reproduction. The identification of the *DAZ* family has led to research into genetic causes of infertility, and expansive work into understanding how the *DAZ* family regulates fertility.

RNA-binding proteins are abundant during spermatogenesis, largely involved in post-transcriptional regulation. During spermatogenesis, extensive translational regulation is used to control the proper timing of differentiation, particularly during spermiogenesis, the differentiation of round spermatids into mature sperm (reviewed in ref. 11 and 12). Many genes are transcribed several days before translation occurs, necessitating a network of mRNA storage and translational control. In addition to mRNA regulation, multiple species of non-coding small RNAs have been identified in the testis. These include miRNAs, piRNAs and MSY-RNAs, though how they intersect with translation regulation and sperm differentiation is unclear.[13-18] Some of this RNA storage and control has been proposed to occur at the chromatoid body, a perinuclear structure most prevalent in round spermatids that contains mRNA, miRNA and several RNA-binding proteins (reviewed in ref. 19). The presence of such a structure and the abundance of multiple classes of small RNAs highlight the importance of RNA binding proteins during spermatogenesis.

The DAZ family of proteins is thought to be involved in the translation activation of mRNA targets.[20,21] In recent years, relevant candidate targets have been identified and the mechanism underlying this regulation is becoming clearer. Additionally, novel roles for *DAZ* family genes in mRNA transport and stability have been discovered. Here we review the functions of the *DAZ* family of genes during spermatogenesis and discuss the various models of their action.

## Evolution of the *DAZ* Gene Family

After the discovery of *DAZ* as a candidate gene for AZFc,[5] the identification of homologs in other species revealed a larger gene family. *DAZ* family genes have a common structure consisting of a RNA-Recognition Motif (RRM) and at least one copy of a motif rich in basic amino acids termed the *DAZ* repeat.[5,6,8,22-24] *Boule* is the ancestral member of the family, and is widely conserved across Metazoa, from the sea anemone through humans (**Fig. 1**).[10,23,25] *Boule* is autosomal and has a single RRM and one DAZ repeat.[8,23,25] The RRM is highly conserved among all *Boule* homologs, with a distinct signature in the RNP1 and RNP2 motifs within the RRM. *Boule* is not found in fungi or plants, indicating that the *DAZ* family is an animal specific family of reproduction genes.[10]

*Dazl* arose from a duplication of *Boule* during vertebrate evolution (**Fig. 1**).[8,10] *Dazl* homologs are also autosomal with only one RRM and one DAZ repeat,[6,9,22,24] and are distinguishable from *Boule* homologs by unique sequences in the RNP1 and RNP2 motifs.[8,10]

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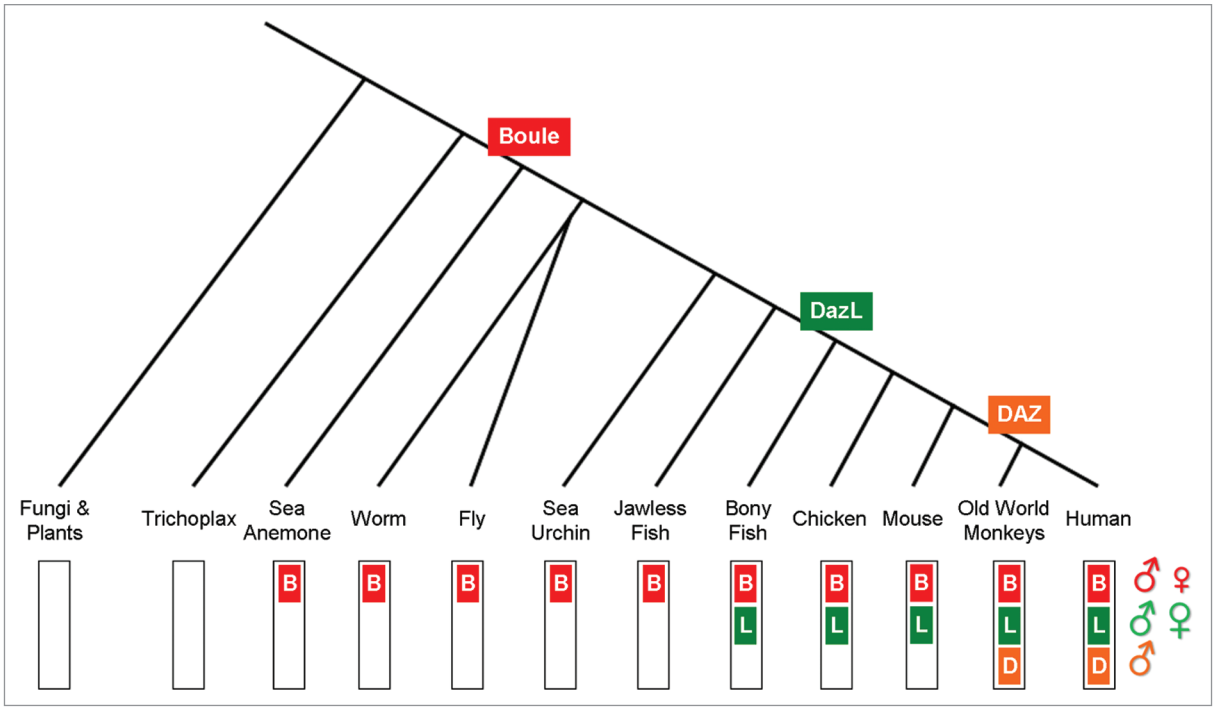


**Figure 1.** *DAZ* family evolution. *Boule* is the ancestral member of the family, and is conserved from the sea anemone through humans, but is not present in Trichoplax, fungi or plants. A duplication of *Boule* during early vertebrate evolution led to *Dazl*. *Dazl* was then duplicated and transposed onto the Y chromosome in the evolution of old world monkeys. It further expanded into a cluster of multiple *DAZ* genes in the evolution of the human lineage. Symbols at right indicate sex-specific roles. *Boule* has predominantly testis functions with occasional ovarian roles, *Dazl* functions in testes and ovaries, while *DAZ* is testis-specific.



*Dazl* arose around the time of vertebrate radiation, and homologs are conserved from bony fish through humans,[6,9,10,22,24,26-28] but are not present in cartilaginous or jawless fish.[10]

During primate evolution, a duplication and transposition of *Dazl* onto the Y chromosome led to *DAZ* (**Fig. 1**).[6,7,29] Subsequent duplication and gene pruning led to four *DAZ* genes in two clusters, each with multiple numbers of DAZ repeats and two with duplications of the RRM.[30] The number of DAZ repeats among the *DAZ* genes is polymorphic both between and within individuals.[30,31] *DAZ* homologs are only present in humans and catarrhine primates (old world monkeys).[7,24,29,32]

Surprisingly, sequence analysis has shown that the presence of *DAZ* has had little effect on either *Dazl* or *Boule* gene evolution in primates, indicating strong functional constraint on these two genes.[33] *DAZ* itself has a higher rate of genetic changes,[34] but neither nonsense nor frameshift mutations affecting the ORF have been detected, suggesting positive selection on *DAZ*.[35] *Dazl* homologs have a higher rate of change than *Boule*,[33] while *Boule* homologs have been shown to be under purifying selection.[10] Indeed, no polymorphisms within the *Boule* coding region were detected among more than 200 fertile and infertile men examined in two different studies,[36] further indicating a strong functional constraint. Such a high level of conservation is rare among reproductive genes, suggesting that *Boule* has an essential germ cell role in animals. Similarly, the continued maintenance of multiple gene duplications suggests that all *DAZ* family genes are critical regulators of fertility.

## *DAZ* Family Gene Expression

Though each *DAZ* family gene has a unique expression pattern, the whole family is restricted to germ cells in nearly all animals. Despite the presence of newer members *Dazl* and *DAZ*, reproduction-specific expression has been preserved for all three *DAZ* family genes. While there is some species specific expression for each *DAZ* family homolog, each gene has maintained the same general pattern across species. Gene families may often show similarities in expression among species, but such clear conservation of homolog-specific expression is unusual. This phenomenon allows a composite picture of common RNA and protein expression to be constructed for each *DAZ* family homolog, summarized in **Figure 2** (red and green lines, respectively). This summary view does not represent the data from any single species, but rather the common expression patterns seen in multiple organisms. Data from specific species is discussed below, with a focus on each homolog's common pattern of expression.

At least one DAZ family homolog is expressed in nearly every stage of spermatogenesis. Though spermatogenesis begins when spermatogonia differentiate, it can be traced back to the differentiation of a subset of embryonic stem cells (ESCs) into primordial germ cells (PGCs). These migrate to the embryonic gonad and become gonocytes (also called prospermatogonia), which proliferate further and eventually become spermatogonia, containing the adult stem cell population. Spermatogonia proliferate further and give rise to primary spermatocytes, which undergo meiosis

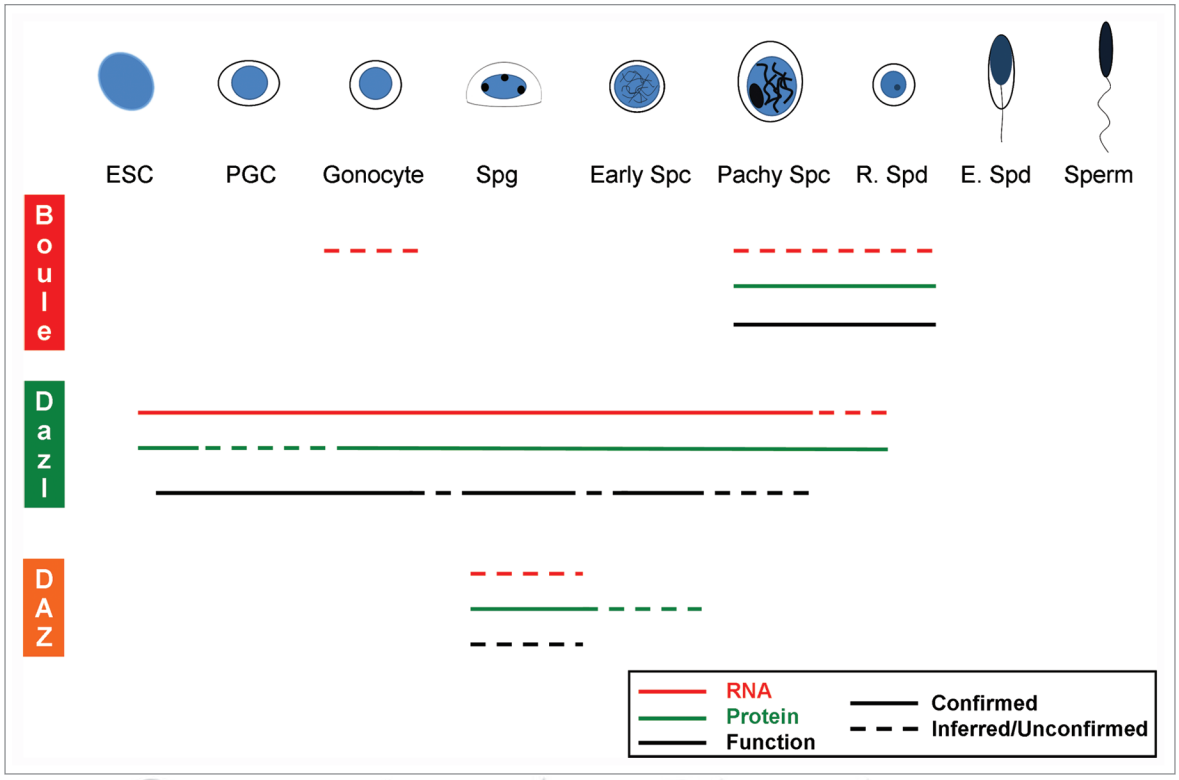


**Figure 2.** Common expression and functions of *DAZ* family genes. Data from multiple species are combined to present a picture of common expression patterns and functions for each *DAZ* family gene during spermatogenesis. A schematic of different steps of germ cell specification and spermatogenesis is shown at top and mRNA expression (red lines) and protein expression (green lines) relative to these steps is shown for each gene. Black lines represent steps in spermatogenesis where each gene is known to have a function. Solid lines represent data confirmed in at least two studies, while dashed lines are either unconfirmed, or inferred from known expression or function data. *Boule* protein is present in pachytene spermatocytes through round spermatids (solid red line) and functions in both meiosis and spermiogenesis (solid black line). *Boule* mRNA is presumed to be present (dashed red line) in cells with Boule protein. *Dazl* homologs are expressed continuously from embryonic stem cells through round spermatids (red and green lines) and are known to function in ESCs, PGCs, gonocytes, spermatogonia and early spermatocytes (solid black lines). *Dazl* likely functions in other cells where it is expressed (dashed black line), but this has not been shown. *DAZ* is known to be expressed in spermatogonia (solid green line) and presumably functions in those cells, though it has not been shown (dashed black line). For details, see text. ESC, embryonic stem cell; PGC, primordial germ cell; Spg, spermatogonia; Early Spc, leptotene/zygotene spermatocyte; Pachy Spc, pachytene spermatocyte; R. Spd, round spermatid; E. Spd, elongating spermatid.

to produce haploid round spermatids. Through a process called spermiogenesis, round spermatids undergo dramatic morphological changes and first become elongating spermatids, and are finally released from the testis as mature spermatozoa.

*Boule* homologs are predominantly transcribed in the testes of fruit flies, sea urchins, chickens, mice and primates,[10] though mRNA expression is reported in the ovaries of *C. elegans*, medaka fish and at low levels in mice.[10,25,37,38] However, in these three species, testis transcription is also observed. Furthermore, since the only instance of Boule protein in ovaries is in *C. elegans*,[37] *Boule* transcription in the ovaries of fish and mice may not lead to protein. Similarly, we have seen low levels of *Boule* mRNA in embryonic gonocytes in mice,[10] but do not detect protein (our unpublished observations). Additionally, *boule* mRNA is detected in the brains of flies,[39,40] but this has not been reported in any other animals.

In the testis, Boule proteins are first present in mid-pachytene spermatocytes and remain through metaphase spermatocytes, with peak levels occurring just before metaphase of meiosis. Boule protein then persists into round spermatids, but is gone by the time elongation begins (**Fig. 2**, Boule green line).[8,41,42] This is true in flies, mice and humans.[8,41] Cell-type specific *Boule* mRNA expression has only been examined in medaka fish, where it is similarly present in both spermatocytes and spermatids.[38] This mRNA pattern is likely the same in both flies and mammals, given the similar protein expression patterns, but it has not been confirmed.

*Dazl* homolog expression has diverged from *Boule*, and homologs are expressed in both males and females in all species so far examined.[10] *Dazl* homologs are initially expressed in ESCs through PGC specification in frogs, fish and mammals (**Fig. 2**, Dazl red and green lines). In Xenopus, *Xdazl* mRNA and protein are both present in the embryonic germ plasm,[26,43] and zebrafish *zDazl* mRNA is detected in the vegetal pole of embryos,[28] a region that later gives rise to germ cells.[44] Similarly, mouse *Dazl* mRNA and human *DAZL* mRNA and protein are present in ESCs through PGCs.[45-47] Together, these expression data indicatethe common expression of *Dazl* during PGC determination of early embryogenesis.

After germ cells are specified, *Dazl* homologs continue to be expressed in gonocytes, through spermatogonia and spermatocytes, and into early round spermatids (**Fig. 2**). Such protein expression is seen in humans, mice and frogs,[8,48-50] while underlying mRNA expression has been confirmed in mammals only through pachytene spermatocytes.[22,51-53] Though reports of post-meiotic Dazl expression in mammals have differed,[8,33,48] we have detected Dazl protein in both mouse and human spermatids (our unpublished observations), and Dazl is present post-meiotically in Xenopus.[50] Additionally, a transgenic reporter driven by the Dazl promoter in mice has shown a similar transcription pattern.[54] Furthermore, one study found human DAZL protein in sperm tails,[55] but this result has not been repeated. Such discrepancies in the reports of Dazl expression are likely due to the use of different antibodies recognizing varying antigens that are differentially accessible during spermatogenesis. Despite the absence of confirmed protein and mRNA data at each stage and slight variations in species-specific Dazl expression,[32] a common pattern of continuous Dazl expression from ESCs through haploid spermatids is clear (**Fig. 2**).

While several papers have reported *DAZ* gene expression, a consensus pattern is not yet clear. In addition to differing antibodies, cross-reactivity with Dazl proteins was sometimes unavoidable. Habermann et al. found DAZ2 protein in mature sperm tails,[56] but this was not repeated in two other studies.[48,57] Using several antibodies to control for cross-reactivity with Dazl, Reijo et al. showed that DAZ is present only in spermatogonia and spermatocytes, with rare expression in spermatids.[48] DAZ protein has been confirmed in human spermatogonia, though not in spermatocytes (**Fig. 2**, DAZ green line).[57] All four *DAZ* genes are transcribed in humans,[57] further complicating expression studies of *DAZ*.

Interestingly, though DAZ family proteins are predominantly present in the cytoplasm, occasional nuclear localization is also observed. In flies, Boule protein is initially in the nucleus of early spermatocytes, and then transits to the cytoplasm just before metaphase,[41] though this is not seen in mammals.[8] Similarly, human and mouse Dazl is nuclear in gonocytes,[48,58] and may translocate from the nuclei of spermatogonia into the cytoplasm of spermatocytes.[48] This translocation of Dazl has not been confirmed, but nonetheless raises an intriguing question of why DAZ family proteins localize to the nuclei of certain cell types. DAZ family proteins may sequester certain transcripts in the nucleus, or could be involved in mRNA processing. However, nuclear localization of Drosophila Boule is dispensable for its function, raising the possibility that Boule is simply stored in the nucleus prior to its function in the cytoplasm.[41] However, the common presence of Dazl in the nuclei of spermatogonia in multiple species suggests important functionality. What this role is remains to be seen, but will likely be important in fertility.

## Functions of DAZ Genes during Spermatogenesis

Because of the broad evolutionary conservation of the *DAZ* family, the functions of *DAZ* genes have been examined in a number of species, revealing requirements for *DAZ* family genes at multiple points during spermatogenesis (summarized in **Fig. 2**, black lines). *DAZ* is known to be important in human spermatogenesis since its deletion is associated with azoospermia.[5] However, causative point mutations in infertile men have yet to be identified, and some *DAZ* deleted men still produce low levels of sperm.[59] Indeed, men with *DAZ* deletions have fathered children both through the use of reproductive technologies[60,61] and though rare, naturally,[3,62] indicating that *DAZ* is not absolutely required for spermatogenesis. However, the presence of *DAZ* within the AZFc region as well as studies detailed below about spermatogenesis requirements of other *DAZ* family members strongly suggest that *DAZ* plays a critical role in normal spermatogenesis.

In accordance with its broad expression pattern, *Dazl* has been shown to have multiple roles throughout spermatogenesis. In frogs and mice, *Dazl* is initially important for PGC proliferation and development. Knockdown of Xenopus *Xdazl* leads to few surviving PGCs, and those that do survive fail to migrate.[43] Similarly, *Dazl* knockout mice have few germ cells that survive into the adult, in both males and females.[49] This defect is first evident at the gonocyte stage in embryonic testes. In a mixed genetic background, *Dazl* null testes are sparsely populated with germ cells by embryonic day 19 (E19),[49] while increased germ cell apoptosis is seen by E14.5 in a pure C57/Bl6 background.[63] Additionally, *Dazl* null ESCs fail to differentiate to PGCs in vitro, while PGCs in vivo fail to properly erase genomic methylation marks.[45] These defects together indicate a problem in PGC development and differentiation, similar to those seen in Xenopus. Though few PGCs are present in *Dazl* null mice and frogs, the presence of germ cells indicates that germ cell specification is occurring in the absence of *Dazl*. However, the in vitro ESC differentiation defect may hint at a role in germ cell specification.

Further studies on *Dazl* null mice on a mixed genetic background have shown additional roles for *Dazl* in both spermatogonia and early spermatocytes. Though most germ cells are absent at birth, some $A_s$ (A-single) and $A_{pr}$ (A-paired) spermatogonia survive, but most do not progress beyond the $A_{al}$ (A-aligned) stages, revealing a function for *Dazl* in spermatogonia differentiation.[64] The few cells that do pass this block are able to enter meiosis, but synaptonemal complexes necessary for homologous recombination fail to form in postnatal day 19 (P19) knockout mice, and spermatocytes cannot progress beyond leptonema.[65] Null germ cells in the pure C57/Bl6 background fail to induce meiosis genes in response to the meiotic signal retinoic acid, showing a further requirement for *Dazl* at the onset of meiosis.[66] This range of defects is specific to germ cells, as wild type spermatogonia can colonize and repopulate a *Dazl* null testis.[67] Taken together, *Dazl* has functions during PGC development and migration, spermatogonia differentiation and the onset and progression of meiosis (**Fig. 2**). *Dazl* presumably also functions between these known steps, corresponding to known expression, but explicit demonstrations of such roles have not yet been shown.

*Boule* functions complement those of *Dazl*, and homologs are important for meiotic division and spermatid differentiation. In Drosophila, *boule* mutant flies have a male-specific arrest at pachynema, prior to metaphase.[23] However, meiosis completes normally in *Boule* knockout mice, and haploid round spermatids

are abundant.[42] Instead, there is a global arrest at step 6 of spermiogenesis, with varying defects in acrosome biogenesis and a complete lack of elongating spermatids.[42] Despite the lack of a meiosis phenotype in *Boule* null mice, *Boule* regulation of meiosis is likely conserved among animals. A pachytene arrest similar to that seen in flies occurs in *C. elegans* with a mutation in the *Boule* homolog *daz-1*, but only in females.[25] In addition, a human *BOULE* transgene can restore meiosis in *boule* mutant flies,[68] and a lack of BOULE protein has been associated with meiotic arrest in men.[36] We therefore proposed that *Dazl* and *Boule* redundantly regulate the progression to meiotic metaphase in mice, and that *Dazl* can compensate for the loss of *Boule* in spermatocytes.[42] While this model has not yet been tested, the accumulating evidence suggests that *Boule* regulation of meiosis is conserved in mammals.

Additionally, though knockout mice revealed a novel role for *Boule* in spermatid differentiation, this function may also be present in flies. In *boule* mutant flies, it was noted that the pachytene-arrested spermatocytes did not differentiate,[23] a phenotype not common to other meiosis-arrest mutants. For example, flies with a mutation in the putative Boule target, *twine*, have a similar meiotic arrest, but many meiosis-arrested spermatocytes in those flies begin to elongate.[69,70] Since differentiation was also disrupted in Drosophila *boule* mutants, this suggests that the spermiogenesis function of *Boule* is also conserved.

Regardless of which specific spermatogenesis roles are conserved, the male-fertility requirement of *Boule* is the same between flies and mice. Despite about 600 million years of evolution separating mice and flies, *Boule* mutations in both species lead to a complete lack of sperm due to a global arrest in spermatogenesis, and the presence of similar-looking multinucleate cysts in the testis (**Fig. 3**). Furthermore, these testes defects are the only phenotype reported in *Boule* null animals of either species,[10,23,39,40,42] highlighting the conservation of a male fertility requirement of *Boule*.

Many experiments have shown a remarkable ability of the *DAZ* family genes to functionally replace each other. Both human *DAZ* and *DAZL* can partially restore germ cell numbers in *Dazl* null mice, though the rescue was moderate and variable among animals.[71,72] These experiments showed that human *DAZ* can function during mammalian spermatogenesis, despite the lack of direct evidence that *DAZ* is necessary for human spermatogenesis. Interestingly, Xenopus *Xdazl* can restore meiosis in *boule* mutant flies,[26] similar to the human *BOULE* rescue discussed above,[68] further supporting the model of *Boule* and *Dazl* redundancy during mammalian meiosis.[42]

Finally, all three *DAZ* family genes have been shown to enhance human ESC differentiation into germ cells in vitro, with overexpression of each *DAZ* family gene alone or in combination leading to varying degrees of enhancement.[46] When all three were expressed together, ESCs were able to differentiate into germ cells with molecular features of spermatids, highlighting the wide range of functions *DAZ* family genes play in spermatogenesis. A similar transient overexpression of *Dazl* in mouse ESCs was also able to promote germ cell differentiation.[73] These ectopic expression studies may not reflect in vivo functions, however. For example, *Boule* overexpression enhanced PGC differentiation in XX (female) ESCs, but not XY (male) ESCs.[46] However, *Boule* expression has not been reported in ESCs of either sex, and *Boule* null female mice have no germ cell defects.[10,42] While these experiments suggest in vivo roles for the ESC-expressed *Dazl*, the results for *Boule* and *DAZ* underscore the ability for compensation among the *DAZ* family when expressed in the right time and place. Together with the data that different homologs can functionally replace each other, the similar in vitro results for each *DAZ* family gene suggest that all *DAZ* family genes can function through similar mechanisms.

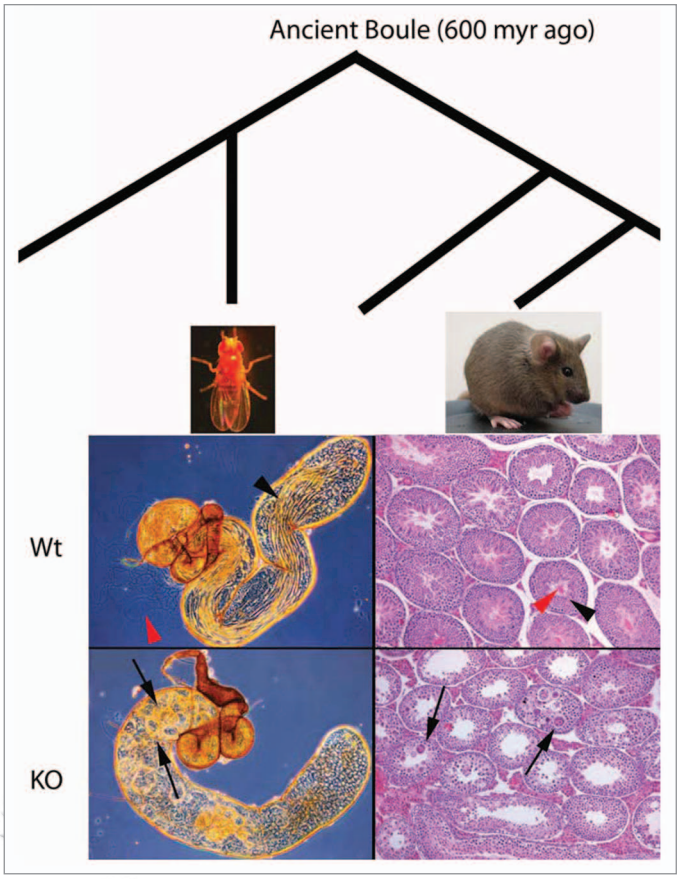


**Figure 3.** *Boule* testis function is conserved in flies and mice. Despite the wide divergence of flies and mice, *Boule* mutations in each species leads to male-only sterility due to a global arrest of spermatogenesis. Mature sperm (red arrowhead) and elongating spermatids (black arrowhead) are abundant in the testis tube of wild type flies, and for the wild-type mouse testis section. Mature sperm (red arrowhead) are seen in the lumen of seminiferous tubules and elongating spermatids are often located next to the lumen (black arrowhead). However both mature sperm and elongating spermatids are completely absent in *Boule* knockout testes of both species. Multinucleate cysts are prevalent throughout null testes of both animals (arrows), further highlighting the conserved spermatogenesis function of *Boule*. Fly testes are from 1-day-old males and images taken at 10x. Mouse testes are from 3-month-old mice and images taken at 10x. Myr, million years.

## Candidate RNA Targets for DAZ Family Proteins

Though many functions of *DAZ* family genes are known (discussed below), relevant and validated RNA targets are less clear.

**Table 1.** Candidate in vivo mRNA targets

| Gene | Candidate target | Motif bound | References |
|---|---|---|---|
| Fly boule, Zebrafish zDazl | twine | GUUC | 74, 77 |
| Mouse Dazl | Cdc25c 5′ UTR | $GU_7GU_{10}GU_{10}GU_7$ | 76 |
| | Cdc25a 3′ UTR | U/AA/GUUC/UAGUAU/AAANAACUUUG/UGAAU/AUG/A | 78 |
| | Mvh | UUCUUCUGUUCUU | 81 |
| | Sycp3 | $U_6GU_3GU_3GU_4$ | 81, 82 |

Though many mRNA targets have been reported, the in vivo candidates shown fulfill three criteria: (1) demonstrated in vivo binding, (2) reported translational defect in *Boule* or *Dazl* null animal model, (3) functional relevance to *Boule* or *Dazl* null phenotype.

Drosophila *boule* enhances translation of a Lac-Z reporter carrying the 3' UTR of the *Cdc25* homolog *twine* in vivo, suggesting that the fly germ cell-specific *Cdc25* homolog is a downstream target of *Boule*.[74] In addition, a *twine* transgene with a *tubulin* 3' UTR was able to rescue meiosis in *boule* mutant flies, indicating that *twine* translation is absent in mutants. Though this model nicely explains the observed meiosis arrests in both Drosophila and *C. elegans*,[8,25,74] no direct interaction between any *Boule* and *Cdc25* homologs has been shown.

Subsequent research into targets has focused on *Dazl* homologs. In homopolymeric binding studies, frog, mouse and human *Dazl* preferentially bind polyU RNA.[26,75] A SELEX approach identified a $(G/CU_n)_n$ motif which is found in the 5'UTR of mouse *Cdc25c*,[76] and zebrafish *zDazl* binds to GUUC, a site found in the 3'UTR of Drosophila *twine* RNA.[77] Using GST-Dazl bound to a column, a 26 bp motif was identified that is present in the *Cdc25a* 3' UTR.[78] Another study identified targets bound by both Dazl and Pumilio2 (Pum2),[79] an RNA-binding protein shown to interact with Dazl.[80] Follow-up studies confirmed *Dazl* binding to a U-rich motif in *Sdad1* mRNA, another cell cycle regulator first identified in yeast.[79]

However, despite multiple reports of candidate targets, there is no overlap between lists, and a discrepancy in the Dazl binding site, perhaps due to the in vitro approaches used in these experiments. Binding conditions are unlikely to match those found in vivo, mRNAs normally in separate cells from Dazl are inappropriately brought together, or legitimate targets may be tightly bound by endogenous protein, therefore preventing their binding to Dazl in vitro. To determine in vivo targets, Reynolds et al. used endogenous immunoprecipitation from whole mouse testes followed by a microarray on co-precipitating RNA, and identified 15 targets with high confidence.[81] Targets were further validated by IP followed by RT-PCR on UV-crosslinked testes to reduce non-specific interactions. Dazl binding to *Mvh* (*Mouse vasa homolog*) and *Sycp3* (*Synaptonemal complex protein 3*) has been confirmed, and translation defects for both of these targets occurs in *Dazl* null animals.[81,82]

Additionally, the presence of the proposed binding sites in the 15 target genes was analyzed.[81] The initial 26 bp motif[78] was only found in six targets, and was not present significantly more than predicted by chance.[81] The SELEX defined $(G/CU_n)_n$ motif, however, was statistically over-represented in the 3'UTRs of targets, and was found to be in evolutionarily conserved regions of the transcripts. This motif was also found in the eight targets previously reported by Jiao et al.[78,81] providing strong support for a common $U_{2\text{-}10}(G/C)U_{2\text{-}10}$ binding motif among Dazl targets. It is not known how prevalent this motif is among testis transcripts, but the flexible nature of this motif suggests that Dazl may bind a wide range of mRNAs.

Notably, using in vivo UV-crosslinking followed by IP and RT-PCR, Reynolds et al. failed to detect Dazl binding to *Prm2*,[81] a target identified by an in vitro screening method.[79] This experiment showed that while in vitro binding studies can correctly uncover a particular binding motif, the specific targets identified may not be relevant in vivo. Therefore, while multiple studies have examined targets of Dazl, only a few candidates have in vivo significance.

The most promising in vivo targets are *Mvh*, *Sycp3* and *Cdc25* homologs (**Table 1**). Three reports showing Dazl binding to *Cdc25* homologs[76-78] together with meiosis rescue studies in flies[26,68,74] is strong evidence for in vivo *Cdc25* binding. Mice have three *Cdc25* genes,[83,84] and Venables et al. only detected binding to *Cdc25c*,[76] while Jiao et al. could only detect binding to *Cdc25a*.[78] Both of these *Cdc25* genes are abundantly expressed in the testis,[83,84] and whether the differential binding is due to the different techniques used, or represents artificial binding due to the in vitro systems remains to be seen.

*Mvh* and *Sycp3* were both identified by the in vivo approach,[81] and are related to known *Dazl* functions. *Mvh* is highly expressed in all germ cells, similar to *Dazl,* and *Vasa* homologs have conserved roles in PGC differentiation.[85] In addition, the male sterile phenotype in *Mvh* knockout mice is due to a final arrest at zygonema,[85] close to the reported leptotene arrest seen in mixed background *Dazl* null mice.[65] Similarly, *Sycp3* is an essential part of the synaptonemal complex that forms during the early stages of meiosis, a time when *Dazl* has been shown to function.[45,65,66] A demonstrated in vivo interaction, translation defects in knockouts and relevance to the observed phenotype together make these genes the best candidate targets so far reported, though none of these targets are a "magic bullet" that explains the primary *Dazl* null phenotype. Similar physiologically relevant data are needed for other reported targets in order to confirm their in vivo regulation by *Dazl*.

Using a similar in vivo immunoprecipitation approach, we have identified the first candidate targets for Boule in mice (VanGompel and Xu, in preparation). We were able to detect interactions between Boule and *Prm1* and *Prm2* mRNAs, genes important for round spermatid differentiation. While not a complete list, these targets are directly relevant to the major phenotype of spermiogenesis arrest in *Boule* null mice.

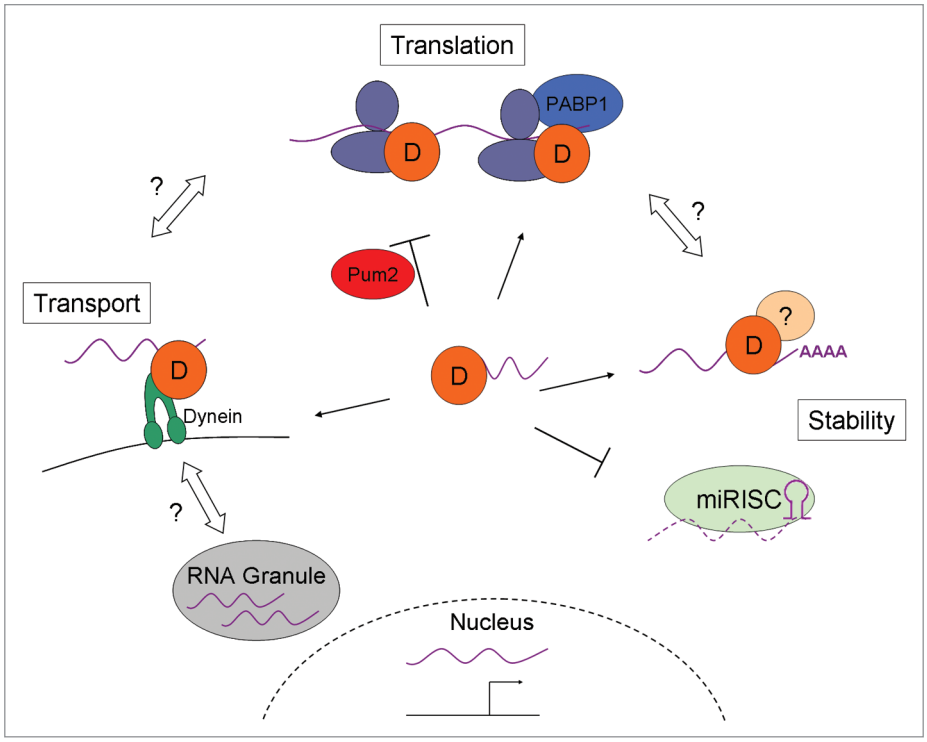


**Figure 4.** Model for *DAZ* family gene functions. DAZ family proteins likely function through similar mechanisms and a composite model representing known cytoplasmic functions of DAZ family proteins is presented. A generic DAZ family protein (orange circle labeled "D") bound to RNA is represented in the middle of the figure. DAZ family proteins have multiple functions, likely dependent on which protein partner they are bound to. Binding to PABP1 promotes association with ribosomes and translation, while binding to the repressor Pum2 inhibits translation. Interactions with Dynein may mediate transport of mRNA targets. Where mRNA is transported is unknown, but DAZ family proteins may transport targets to and from RNA granules, such as the chromatoid body, or to polysomes for translation activation. DAZ family proteins may promote mRNA stability either through the inhibition of miRNA-mediated degradation, or the promotion of polyadenylation through binding to an unknown factor (beige circle labeled "?"). Increased stability may enhance translation, or vice versa. Solid lines represent known mechanisms, while open double-sided arrows represent speculated links between known roles.

## The DAZ Family as Translational Activators

Most studies have focused on the *DAZ* family as translational activators. This model was first established through the studies in Drosophila discussed above, implicating *boule* in the translational regulation of *twine*.[74] Similarly, zebrafish *zDazl* can stimulate translation of a luciferase reporter fused to the *twine* 3' UTR in cell culture.[77] Using a tethered assay in Xenopus oocytes in which proteins were forced into proximity of reporter mRNAs, Dazl homologs stimulated translation through enhanced recruitment of 80S ribosomes.[86] This translation activation was dependent on an interaction with Poly(A) Binding Protein 1 (PABP1), but still occurred in the absence of poly(A) tails on reporter constructs. This led to a model in which Dazl recruits PABP1 to mRNAs in the absence of an adequate poly(A) tail, and thus promotes translation.[86] This model is particularly intriguing in the context of mammalian spermatogenesis because several transcripts are known to be deadenylated prior to translation.[87,88] Xdazl was similarly shown to activate translation of *RINGO/Spy* mRNA, but in a Pum2 dependent manner.[89] Dazl bound target mRNA, but could not activate translation until the translational repressor Pum2 dissociated from the transcript. This shows that the function of the *DAZ* family is context dependent, and may vary depending on the stage of spermatogenesis and what other proteins are present at any given time (**Fig. 4**). Additionally, while the general mechanisms are likely to be broadly conserved, the specific contexts and players involved may differ among species. Further studies into how other interacting proteins regulate DAZ family function will prove fruitful.

While these studies examined the mechanism of DAZ family function in vitro, others have shown a similar translational role in vivo. Mammalian Dazl is present in active polysomes in mouse testes,[75] and Drosophila Boule enhances the translation of a transgenic reporter through the *twine* 3' UTR.[74] As discussed previously, translation of the two *Dazl* candidate targets, *Mvh* and *Sycp3*, was reduced in *Dazl* null testes, further suggesting that Dazl is a translational activator in vivo.[81,82] Protein of these targets was still detectable in *Dazl* knockouts, however, indicating that Dazl is acting as an enhancer of translation, and not an essential activator as proposed in the *Boule-Cdc25* model. While the combined evidence for the translational regulation of mRNA targets is strong, it is important to note that these data can not yet account for the dramatic loss of germ cells in *Dazl* null mice. Key targets that regulate germ cell numbers may not yet be identified, or alternate functions that have broader effects may cause the observed phenotype.

**Table 2.** DAZ family interacting proteins

| Gene | Partners | Category | References |
|---|---|---|---|
| Boule | Boule, Dazl, DAZ | DAZ Family | 8, 80, 90 |
| | PABP1, Pum2, Cpb-3 | Other RNA-Binding Proteins | 86, 90, 91 |
| Dazl | Boule, Dazl, DAZ | DAZ Family | 8, 58, 80, 90 |
| | PABP1, ePABP, Pum2, hQK3, DAZAP1 | Other RNA-Binding Proteins | 80, 86, 90, 92 |
| | Dynein, Dzip1, DAZAP2 | Non-RNA-Binding Proteins | 80, 92, 94 |
| DAZ | Boule, Dazl | DAZ Family | 58, 80 |
| | PABP1, Pum2, hQK3, DAZAP1 | Other RNA-Binding Proteins | 80, 86, 92 |
| | Dzip1, DAZAP2 | Non-RNA-Binding Proteins | 80, 92, 93 |

## Non-Translational Roles for the *DAZ* Family

In addition to translation activation, *DAZ* family genes have other roles. Several binding partners have been identified (**Table 2 and Fig. 4**), and interactions with other RNA-binding proteins is a common theme. The mammalian proteins can form homo- and heterodimers,[8,58,80,90] further indicating similar functions, and suggesting a possible mechanism for the flexibility in functionality observed in ectopic rescue studies. The translational repressor Pum2 can bind all three DAZ members in humans.[80,90] PABP1 can interact with Boule, Dazl and DAZ homologs from frog, mouse and humans,[86] while Xdazl also interacts with ePABP (embryonic PABP),[86,89] and *C. elegans* DAZ-1 interacts with the CPEB (Cytoplasmic Polyadenylation Element Binding protein) homolog Cpb-3.[91] Both human DAZ and DAZL have also been shown to interact with RNA-binding proteins hQK3 and DAZAP1.[80,92] In addition, novel, non-RNA-binding proteins DZIP1 and DAZAP2 have been identified as binding partners through interaction screens with DAZ and DAZL.[80,92,93] Such a variety of interactions further supports a model of context-dependent DAZ family function, where specific protein partners mediate a range of roles (**Fig. 4**). Indeed, which RNA targets DAZ family proteins are bound to may also depend on the context of other protein partners. The Pumilio family of RNA-binding proteins has been shown to differentially bind RNA targets based on what protein partners they are bound to,[90] and DAZ family proteins may utilize a similar mechanism for binding different targets.

Mouse Dazl was also found to interact with dynein light chain in mouse testes, and can move on the microtubule network in cell culture (**Fig. 4**).[94] In a dynein-dependent manner in vitro, Dazl can transport mRNA carrying putative binding sites, including those found in candidate targets *Tpx-1*, *Cdc25c* and *Mvh*, on microtubules. These mRNAs formed perinuclear aggregates, at structures presumed to be stress granules, where ectopic Dazl also accumulated.[94] Active mRNA transport in male germ cells is not well-studied, but has been reported. The testis specific kinesin KIF17b can shuttle protein-RNA complexes in and out of the nucleus,[95] and also associates with Miwi and the chromatoid body (CB),[96] suggesting transport of mRNA to and from the CB. In other cell types, mRNA is stored in stress granules to protect transcripts from degradation,[97,98] a parallel the authors propose occurs with Dazl-bound targets.[94] Further studies are needed to determine if Dazl transports targets to the CB or other RNA granules in germ cells, and whether other DAZ family proteins are similarly involved in transport.

Could the DAZ family transport RNA for safe storage? If protecting mRNA from degradation is important in spermatogenesis, what is the targeting mechanism? A likely candidate is through specific miRNAs (**Fig. 4**). miRNAs are known to inhibit translation of targets, and this inhibition is often due to miRNA-mediated mRNA degradation.[99] Zebrafish *zDazl* was recently shown to prevent miRNA mediated decay of *nanos1* and *tdrd7* transcripts,[100] though direct binding to these mRNAs was not shown. Using injections into zebrafish embryos, the authors showed that *zDazl* prevents miRNA mediated inhibition of reporters, dependent on the presence of the GUUC binding motif. Furthermore, this motif does not overlap with the miRNA binding site, but was necessary for zDazl to stabilize the mRNA.[100] Since miRNA is present in the chromatoid body, it is an intriguing possibility that DAZ family proteins are either protecting targets from miRNA within the CB, or are involved in transporting them away from miRISC (microRNA Induced Silencing Complex) in germ cells.

While surprising, reduction of mRNA levels of *Dazl* targets has also been reported. Though reduced translation was noted for *Mvh* and *Sycp3* in *Dazl* null mice, transcripts were reduced in postnatal day 5 (P5) null testes, a result that contributed to their identification as targets.[81] This instability was presumed to be a consequence of reduced translation, but a direct stability effect could not be ruled out.[81] Furthermore, quantitative RT-PCR using *Dazl* null embryonic testes has also shown a reduction in mRNA of both of these targets.[45,66] Those experiments focused on the ability of *Dazl* null germ cells to respond to meiosis signals, so the reduction was noted only as a failure to initiate meiosis. Additionally, a microarray study on P7 wild type and *Dazl* null testes found a large number of transcripts that were reduced in knockouts.[101] A similar result was obtained in a human microarray study on men with *DAZ* deletions.[102] These studies hint at a potential role for the *DAZ* family in maintaining RNA levels, though a direct role for this in vivo has not yet been shown. Determining if reductions in transcript levels are due to a direct loss of *DAZ* family genes will clarify these new data.

Finally, in the zebrafish miRNA study described above, zDazl induced polyadenylation of transcripts, a novel function for the *DAZ* family. This polyadenylation was independent of translation, indicating that mRNA stability is independent from

translation activation. Furthermore, polyadenylation may be an alternate method of PABP recruitment and subsequent translation activation. How zDazl mediates polyadenylation is not known, but it is likely through an as yet unidentified binding partner (**Fig. 4**). Cytoplasmic polyadenylation is well described during oogenesis (reviewed in ref. 103), and is beginning to be appreciated in spermatogenesis.[104] In one well-studied mechanism in females, CPEB binds to cytoplasmic polyadenylation elements and recruits the polyadenylation apparatus. As mentioned, *C. elegans* DAZ-1 interacts with a CPEB homolog,[91] suggesting a role for *DAZ* family genes in polyadenylation in worms. In mice, knockout of the testis-specific cytoplasmic poly(A) polymerase *Tpap* leads to a spermiogenesis arrest similar to that seen in *Boule* knockouts.[42,105] Whether Boule and Tpap interact is not known, but the similar knockout phenotypes suggest that they may function in the same pathway, perhaps through regulation of mRNA stability. It is also possible that translation activation is a consequence of increased mRNA stability through polyadenylation, and not a direct function of the *DAZ* family. Determining how *Boule* regulates targets will help determine if such mechanisms are broadly used, and what roles they play in spermatogenesis.

## Conclusion

Recent findings are painting a new picture for *DAZ* family-mediated regulation of targets, beyond the simple model of translation activation. Their roles in translation activation have been well-established using many systems, but likely represent only one of many functions. Specific mechanisms may differ in the broad range of cell types in which this family functions, and *DAZ* family genes may play multiple roles within the same cells. This range of functions may be determined in part by which proteins the DAZ family is bound to at any given time. Yet despite the variety of functions and mechanisms, the DAZ proteins have been highly conserved, and can still functionally replace each other in limited contexts. Such strong selective pressure on reproductive genes is rare, and suggests an essential role for these genes in the germ cells of animals. While possible mechanisms are emerging, why these functions are required in germ cells of all animals, and why humans require more *DAZ* family genes than other species are puzzles that remain. Much work is needed to address these interesting questions.


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