Differential Conservation and Divergence of Fertility Genes *boule* and *dazl* in the Rainbow Trout

Mingyou Li¹, Qian Shen¹, Hongyan Xu¹, Fong Mei Wong¹, Jianzhou Cui³, Zhendong Li¹, Ni Hong¹, Li Wang¹, Haobin Zhao¹, Bo Ma², Yunhan Hong¹,²,³

¹Department of Biological Sciences, National University of Singapore, Singapore, Singapore, ²Heilongjiang Fisheries Research Institute, Ha’erbin City, China, ³Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Jingzhou, China

**Abstract**

**Background:** The genes *boule* and *dazl* are members of the DAZ (Deleted in Azoospermia) family encoding RNA binding proteins essential for germ cell development. Although *dazl* exhibits bisexual expression in mitotic and meiotic germ cells in diverse animals, *boule* shows unisexual meiotic expression in invertebrates and mammals but a bisexual mitotic and meiotic expression in medaka. How *boule* and *dazl* have evolved different expression patterns in diverse organisms has remained unknown.

**Methodology and Principal Findings:** Here we chose the fish rainbow trout (*Oncorhynchus mykiss*) as a second lower vertebrate model to investigate the expression of *boule* and *dazl*. By molecular cloning and sequence comparison, we identified cDNAs encoding the trout Boule and Dazl proteins, which have a conserved RNA-recognition motif and a maximal similarity to their homologs. By RT-PCR analysis, adult RNA expression of trout *boule* and *dazl* is restricted to the gonads of both sexes. By chromogenic and two-color fluorescence in situ hybridization, we revealed bisexual and germine-specific expression of *boule* and *dazl*. We found that *dazl* displays conserved expression throughout gametogenesis and concentrates in the Balbiani’s body of early oocytes and the chromatin body of sperms. Surprisingly, *boule* exhibits mitotic and meiotic expression in the male but meiosis-specific expression in the female.

**Conclusions:** Our data underscores differential conservation and divergence of DAZ family genes during vertebrate evolution. We propose a model in which the diversity of *boule* expression in sex and stage specificity might have resulted from selective loss or gain of its expression in one sex and mitotic germ cells.

**Citation:** Li M, Shen Q, Xu H, Wong FM, Cui J, et al. (2011) Differential Conservation and Divergence of Fertility Genes *boule* and *dazl* in the Rainbow Trout. PLoS ONE 6(1): e15910. doi:10.1371/journal.pone.0015910

**Editor:** Leo T. O. Lee, University of Hong Kong, Hong Kong

**Received** September 23, 2010; **Accepted** November 29, 2010; **Published** January 6, 2011

**Copyright:** © 2011 Li et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the Biomedical Research Council of Singapore (R-08-1-21-19-585 & SBIC-SSCC C-002-2007). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: dbshyh@nus.edu.sg

These authors contributed equally to this work.

**Introduction**

Gametogenesis, oogenesis in the ovary and spermatogenesis in the testis, proceeds in multiple processes including mitotic proliferation of germ stem cells and meiosis, as well as post-meiotic spermiogenesis in male. The DAZ (Deleted in Azoospermia) gene family represents one of the few lines of evidence for evolutionary conservation of these processes at the molecular level [1]. This family comprises *daz*, *dazl*, and *boule*, which encode RNA-binding proteins characteristic of a conserved RNA recognition motif (RRM) and one or multiple repeats of the DAZ motif. The founder member of the DAZ family is the human Daz gene that forms a cluster on the Y chromosome [2,3]. Daz has been thought of as being a male fertility factor, as deletion of the Daz cluster is capable of rescuing the *boule* meiotic entry phenotype in *Drosophila* as a gene essential for the germline development [12]. Similarly, the nematode *Caenorhabditis elegans* has a *boule* gene although it was initially called *dazl-1* [13]. The functional conservation between *dazl* and *boule* was demonstrated by the fact that transgenic expression of the *Xenopus dazl* was capable of rescuing the *boule* meiotic entry phenotype in *Drosophila* [8].

The DAZ family genes all show a conserved germ cell-specific expression [1]. They also share a conserved function in germline development from invertebrates to mammals, as demonstrated by the fact that the human Dazla can rescue the *Drosophila boule* mutant to some extent [14]. Since both *boule* and *dazl* exist in vertebrates, and only *boule* is present in invertebrates, *boule* has been hypothesized to be the ancestor of the Daz cluster, via its duplication and transposition to the Y chromosome during the evolution of primates [3]. The Dazl homolog has been found also in all major groups of non-mammalian vertebrates including chicken [7], *Xenopus* [8], axolotl [9], zebrafish [10], and medaka [11]. The third family member *boule* was identified in *Drosophila* as a gene essential for the germline development [12].
According to this hypothesis, boule underwent gene duplication during vertebrate evolution, generating the autosomal dazl, which underwent further duplications, producing additional copies that were translocated to the Y-chromosome in primates [1].

On the other hand, the DAZ family genes exhibit a salient evolutionary variation in sex- and stage-specific expression. Specifically, dazl exhibits bisexual expression in diverse species, while the human Daz is specifically expressed in the male germline [1]. Intriguingly, boule expression is generally unisexual and shows considerable variation in opposite sexes across animal phyla: boule is expressed only in the male germline of Drosophila and mammals, whereas the nematode (C. elegans) boule (also called daz-1) is expressed in the female germline [15]. In all these organisms, boule expression is meiotic.

We are interested in the evolution of the DAZ family genes in fish as the most primitive group of vertebrates. Previously, we and others have identified dazl in zebrafish [16], gibel carp [17], medaka and stickleback [18], and boule in medaka and stickleback [18]. So far medaka is the only fish in which the RNA expression patterns of both boule and dazl have been analyzed in detail [18]. Interestingly, in this organism, not only dazl but also boule display bisexual expression in mitotic and meiotic germ cells [11,18], raising a need for examining their expression in more organisms to better our understanding of the origin and evolution of DAZ family genes in vertebrates.

We are interested in the evolution of DAZ gene family in fish. Here we chose the rainbow trout (Oncorhynchus mykiss), which separated ~200 millions years ago from the lineage leading to medaka [19], an evolutionary distance similar to that between human and platypus. The trout is a unique fish species for germ cell biology and biotechnology. In this organism, a vasa homolog has been identified as the first and only germ cell marker [20]. A transgenic trout line has been produced that expresses green fluorescent protein (GFP) from the trout vasa promoter [21] and allows for the isolation of primordial germ cells (PGCs) by fluorescence activated cell sorting from the larval gonad [22]. More importantly, transplantation of trout PGCs or testicular male germ cells into salmon embryos has led to successful surrogate reproduction [23,24]. In this study, we identified boule and dazl in this organism and examined their RNA expression. We show that the trout boule and dazl have differential bisexual germ cell-specific expression and are markers for different stages of male and female gametogenesis.

Results
Gene identification
To identify trout boule and dazl, we exploited molecular cloning and bioinformatics approach. BLAST search by using the medaka sequences against available libraries of trout expressed sequence

Figure 1. Nucleotide and amino acid sequences of the rainbow trout boule and dazl. (A) boule. (B) dazl. Start and stop codons are in bold. Sequences underlined are primer sequenced used for cDNA cloning and RT-PCR analysis, which also flank the regions used for the synthesis of RNA probes for in situ hybridization.
doi:10.1371/journal.pone.0015910.g001
tags led to the identification of two cDNA sequences as the putative trout \textit{boule} (HQ696915) and \textit{dazl} (HQ696914). Both cDNAs were PCR-cloned by using primers complementary to the termini and sequenced. The \textit{boule} cDNA is 806 nt in length, contains a 216-nt 5'-untranslated region (UTR) and a partial open reading frame (ORF) of 558 nt for 195 amino acid residues [25] (Figure 1). The predicted trout Boule displays a maximal identity of 77\% and 54\% to the medaka and mammalian Boule proteins. The \textit{dazl} cDNA is 706 nt, contains a 48-nt 5'-UTR and a full open reading frame (ORF) for 212 aa (Figure 1B). The predicted trout Dazl is maximally 77\% and 54\% identical to the medaka and mammalian Dazl proteins.

The conserved positions between the trout Boule or Dazl and their respective homologs do not evenly distribute along entire molecules but instead reside within the RRM. Therefore, we focused our comparison on a multiple sequence alignment of the RRM only. This revealed that Boule differs from Dazl in 20 invariant and/or conserved positions, besides 27 invariant and/or conserved positions common to both Boule and Dazl (Figure 2A). These conserved positions are the same as those previously identified [10]. On a phylogenetic tree, Boule proteins are clustered together, whereas all Dazl forms a separate clade (Figure 2B). Interestingly, the Boule-Dazl branching coincides with separation between fish and tetrapod lineages (Figure 2C). This, together with the fact that fish Boule is more similar to mammalian Boule than to fish Dazl, strongly supports the early divergence of vertebrate \textit{boule} and \textit{dazl} before fish-tetrapod separation.

\textbf{Bisexual RNA expression}

In many species so far examined except cattle [26], \textit{dazl} has bisexual germline expression. However, \textit{boule} has considerable diversity, ranging from unisexual expression in male fly [12], male mammals [1], but female worm [15] and bisexual expression in medaka [18]. By RT-PCR, the transcripts of trout \textit{boule} and \textit{dazl

![Figure 2. Comparisons of Boule and Dazl proteins between rainbow trout and other organisms.](https://example.com/figure2.png)

(A) Multiple sequence alignment of the RRM. Boule and Dazl proteins share 16 invariant residues (asterisks) and 13 conserved positions (%). There are 21 invariant or conserved residues characteristic of Boule (\&) and Dazl (#) proteins each. (B) Phylogenetic tree of DAZ family proteins. The branching between Boule and Dazl coincides with the branching between the fish and tetrapod lineages, and molecular trees on the basis of Boule and Dazl sequences are in accordance with organism relationships, indicating that generation of \textit{boule} and \textit{dazl} took place in early vertebrate evolution. Followed species are gene accession numbers. (C) Organism phylogeny and branching times of major fish groups and tetrapod vertebrates. Fish timing is according to [19].

doi:10.1371/journal.pone.0015910.g002
were found to be absent in somatic tissues but high in adult gonads of both sexes (Figure 3), suggesting that adult boule and dazl expression may be restricted to germ cells.

Germ cell-specific expression during oogenesis

We performed in situ hybridization (ISH) to examine the boule and dazl RNA expression during gametogenesis and compared with vasa, the well-studied germ cell marker. The adult trout ovary comprises a small number of oogonia and oocytes and oogenesis proceeds in stages I to IV. By chromogenic ISH, the boule RNA was found to be restricted to germ cells but barely detectable in surrounding somatic cells (Figure 4A–C). Notably, it is hardly detectable in undifferentiated oogonia and becomes detectable in differentiating oogonia seemingly entering into meiosis, increases in meiotic female germ cells, namely stage I-III oocytes (Figure 4D and E). In addition, the boule RNA displays a dynamic subcellular distribution relative to stages of oogenesis: It disperses evenly in small oocytes at stage I, concentrates in several patches in growing oocytes at stage II, and re-disperses again in oocytes from stage III onwards (Figure 4A–C).

At lower magnifications, the dazl signal was found to concentrate in speckles at a low magnification (Figure 5A and B). These speckles appear to be hollow-like structures at a higher magnification (Figure 5C). The dazl RNA is also restricted to germ cells and barely detectable in surrounding somatic cells (Figure 5D–G). Unlike boule, the dazl RNA persists throughout oogenesis, with its signal being clearly visible in undifferentiated oogonia (Figure 5D–G). The dazl RNA also displays a dynamic subcellular distribution different from boule. In oogonia, dazl RNA distributes evenly without forming speckles (Figure 5F and G).

Differential expression during oogenesis

After verifying the specificity of both boule and dazl probes by chromogenic ISH, we wanted to precisely compare the RNA expression patterns of boule and dazl together with vasa at different stages of oogenesis. The vasa expression has been demonstrated to germline-specific [20,21]. To this, we developed in the trout a two-color fluorescence in situ hybridization (FISH) procedure. We first compared boule and vasa by FISH (Figure 6). The vasa RNA persists throughout oogenesis. The vasa signal is relatively weak in stage-I oocytes and become most abundant in oocytes at stages II and III (Figure 6A–D). At higher magnification, the vasa signal is easily detectable also in undifferentiated oogonia (Figure 6E and F) and differentiating oogonia seemingly entering into meiosis (Figure 6G–I). The boule signal is visible in early oocytes at stage I, peaks at stage II, declines at stage III and dramatically decreases at stage IV (Figure 6A–D). A closer inspection at a higher

Figure 3. RT-PCR analysis of trout boule and dazl RNA expression. The RNA is abundant in the female gonad ovary and male gonad testis but absent in all somatic tissues examined. doi:10.1371/journal.pone.0015910.g003

Figure 4. Expression of boule RNA during oogenesis. Adult ovarian cryosections were hybridized to antisense RNA probes and the signals were visualized by chromogenic staining. (A) Lower magnification view showing different stages of oocytes (I to IV). (B) Larger magnification of the area framed by solid line in (A), highlighting speckles of boule RNA in stage-II oocytes. (C) Larger magnification of the area framed by broken line in (A), highlighting the absence of boule RNA speckles in oocytes at stage I and III, and the lack of boule signal in somatic cells (asterisks). (D) Oogonia and early oocytes. (E) Larger magnification of the framed area in (D), highlighting differentiating oogonia (og2) and early oocytes at stage I with residual nuclear staining by DAPI (inset). doi:10.1371/journal.pone.0015910.g004
magnification revealed that the onset of detectable boule RNA expression occurred in differentiating oogonia (Figure 6G and H), in which the boule signal is seen as small particles surrounding the nucleus (Figure 6H’ and I’). These differentiating oogonia are characteristic of a larger size (~30 μm in diameter compared to ~20 μm for undifferentiated oogonia) and strong DAPI staining. DAPI does not stain oocyte nuclei. A larger size and remaining DAPI staining suggest that these are differentiating oogonia entering into meiosis. Generally, the vasa signal is stronger than the boule signal except for stage I, where boule is stronger than vasa. Notably, boule and vasa RNA display independent subcellular distribution. This is most evident in stage-II oocytes, where the RNAs of both genes may exhibit co-distribution or alternative distribution (Figure 6).

We then examined the dazl and vasa by FISH (Figure 7). This revealed a similar expression pattern and also independent subcellular distribution for both genes, with some oocytes predominantly showing the dazl or vasa signal at the focus of section/observation (Figure 7A–D). We finally analyzed boule and dazl by FISH (Figure 8). We observed again independent subcellular distribution, predominantly mosaic distribution (Figure 8). Another salient observation was made in stage-II oocytes. In these oocytes, dazl concentrates in a cytoplasmic structure, the so-called Balbiani’s body (BB), where boule is essentially absent (Figure 8). BB is a spherical membrane-less structure, forming in contact with the oocyte nucleus in early oogenesis [27,28,29] and containing mitochondria, endoplasmic reticulum and dazl mRNA during early oogenesis in Xenopus [27], zebrafish [16] and medaka [18]. Therefore, differential concentration in the BB is a conserved divergence between boule and dazl RNAs in trout and other organisms.

Differential expression during spermatogenesis

We furthered our experiments to examine the expression of boule and dazl by FISH during spermatogenesis in the adult testis. The expression of boule and dazl was restricted to male germ cells but absent in surrounding somatic cells (Figure 9). Importantly, both the boule and dazl signal peak in spermatogonia, in sharp contrast to female expression of boule, which is meiosis-specific but absent in oogonia of the mitotic phase. Another difference was seen in postmeiotic spermatids, where the boule signal was hardly detectable, and the dazl signal persisted.

Differential subcellular distribution of boule and dazl is also evident in the tests. In all these spermatogenic cells, the boule distributed evenly in the cytoplasm, whereas the dazl RNA concentrated in speckles in spermatagonia and spermatocytes (Figure 10). Strikingly, in post-meiotic spermatids, the dazl RNA localizes in a structure called the chromatoid body (CB) (Figure 9C and D). CB is a unique membrane-less cytoplasmic structure in spermatids and sperm, which contains germ plasm RNA and protein components such as vasa [29].

Discussion

In the present study, we have identified the trout boule and dazl and analyzed their expression in detail during oogenesis and...
spermatogenesis. Several lines of evidence, including phylogenetic sequence comparisons, protein structure and expression patterns, support the notion that the trout boule and dazl are orthologous to the known boule and dazl, respectively. With the identification of mammalian boule, the DAZ gene family was proposed to have evolved from boule by gene duplication and/or translocation [1]. Our previous work on medaka boule and dazl provided a first direct evidence for ancient gene duplication during early vertebrate evolution, prior to the separation between fish and tetrapod lineages approximately 450 million years ago (Figure 10A). The identification of trout boule and dazl and their expression analyses in this study underscore the notion that boule and dazl coexist widely in vertebrates, and that boule and dazl become paralogs of each other since their initial duplication event in a common ancestor.

In this study, we have developed a two-color FISH procedure for the simultaneous analysis of multiple RNA molecules at a high sensitivity. By using this procedure we revealed several important observations on the differential expression of trout boule and dazl during oogenesis and spermatogenesis. First, both genes exhibit a bisexual germline-specific expression, similar to the vasa, the best studied germ cell marker in diverse organisms including the zebrafish [30,31], medaka [32], gibel carp [33] and trout [20,21]. Therefore, the germline-specific expression of the DAZ family genes has generally been conserved in invertebrates and vertebrates.

Second, the trout dazl shows not only expression in the mitotic and meiotic phases of both female and male gametogenesis but also stage-specific subcellular localization into the Balbiani’s body and chromatoid body, cytoplasmic structures that are thought as being stage-specific germ plasm in the oocyte and sperm [18,29]. This expression pattern and subcellular localization conforms to those reported for its homolog in three other fish species, namely

Figure 6. Expression of boule and vasa transcripts in trout oogenesis. Adult ovarian cryosections were hybridized to antisense RNA probes and the signals were visualized by fluorescence staining. Nuclei were stained blue by DAPI. (A–D) Stages I–IV of oocytes. Oocytes showing co-distribution (*) and alternative distribution (#) are indicated. (E and F) Oogonia. (G–I’) Differentiating oogonium entering into meiosis, highlighting the onset of boule RNA expression at this stage. (H’ and I’) Larger magnifications of framed areas in (H and I). This oogonium retains nuclear staining by DAPI and contains a few speckles of boule RNA (arrows). Scale bars, 50 μm.
doi:10.1371/journal.pone.0015910.g006
medaka [18], gibel carp [17] and zebrafish [30,31], and several representatives of tetrapod vertebrates including *Xenopus* [8], chicken [34] and mammals [1]. Thus, the bisexual mitotic and meiotic germline expression and subcellular localization we revealed for the medaka and trout *dazl* gene must represent the characteristics of the vertebrate *dazl* gene prototype, which have been highly conserved during evolution of vertebrates from fish to mammals.

Finally and most importantly, we have made unusual observations on the trout *boule* expression. In diverse organisms, *boule* is best known for its unisexual meiotic expression and requirement in invertebrates. In vertebrates, *boule* shows considerable variations, ranging from male-only meiotic expression in mammals to bisexual expression in embryonic germ cells and adult germ cells at the mitotic and meiotic phases of medaka oogenesis and spermatogenesis. In this study, *boule* expression is bisexual in trout similar to medaka. In the testis, the trout *boule* also resembles its medaka homolog in weak expression in spermatogonia of the mitotic phase, strong expression in spermatocytes of the meiotic phase and rapid disappearance in spermatids/sperm of the meiotic phase. These suggest that *boule* has evolved meiosis-preferential expression in male germ cells of both trout and medaka. In the trout ovary, however, *boule* expression is absent in undifferentiated oogonia even by sensitive FISH, in contrast to medaka *boule* that shows easily detectable expression together with *dazl* throughout oogenesis including oogonia [18]. Interestingly, trout *boule* commences expression in differentiating oogonia seemingly entering into meiosis and dramatically increases in the early meiotic phase as seen in oocytes at stages I and II. It is these early oocytes that express an even higher level of RNA for *boule* than *dazl*. Moreover, meiosis progression into stages-III oocytes accompanies a decrease in *boule* expression. In addition, the trout *boule* resembles its medaka homolog in the absence of localization into the Balbiani’s body and chromatoid body. These data demonstrate that female *boule* expression in trout is meiotic and occurs predominantly in the early meiotic phase. Therefore, the present work provides two new germ cells markers to identify different stages of gametogenesis in trout for germ cell biology and reproductive technology.

The two-color procedure also led to the observation that the trout *boule*, *dazl* and/or *vasa* exhibit independent subcellular distribution instead of colocalization during gametogenesis, which
is most evident in early oocyte development. Throughout spermatogenesis, *boule* diffuses and *dazl* forms speckles. During oogenesis, *boule* also diffuses and concentrates in speckles in stage-II oocytes, whereas *dazl* localizes in many speckles of hollow-like structures again in stage-II oocytes. This observation in trout further adds the diversity to the expression of DAZ family genes.

Our detailed comparative expression analyses of the DAZ family genes in medaka [11,18] and trout (this study) as two representatives of primitive vertebrates shed important lights on the evolution of this family in vertebrates (Figure 10A). The meiotic-specific female expression plus meiosis-preferential male expression of trout *boule*, and meiosis-preferential bisexual expression of medaka *boule*, together with the highly conserved bisexual expression of *dazl* throughout gametogenesis in diverse species, point to differential conservation and divergence of the DAZ family genes in vertebrates. We propose two alternative models for the evolution of differential *boule* and *dazl* expression in diverse species (Figure 10B). In a loss of expression model, the common ancestral gene had bisexual mitotic and meiotic expression, and the ancient gene duplication generated *boule* and *dazl*, which were initially identical in bisexual expression but subsequently underwent different ways of evolution: *dazl* shows conservation, whereas *boule* exhibits divergence towards unisexual meiotic expression by loss of mitotic expression, loss of female expression and loss of subcellular localization, ultimately leading to meiotic male *boule* expression as seen in mammals. According to this loss of expression model, *boule* has evolved by selective loss of expression, whereas *dazl* has conserved the original expression. A gain of expression model would also apply to the DAZ family evolution: gain of mitotic expression, gain of female expression and gain of subcellular localization, resulting in *dazl* as seen diverse species. The situations we found in medaka and trout appears to represent two intermediate steps of *boule* evolution and supports either model. Highly conserved bisexual *dazl* expression in mitotic and meiotic germ cells favors the gain of expression model, whereas unisexual meiotic expression of *boule* in invertebrates as the only family member seemingly supports the gain of expression model. Future examination of DAZ family genes in more primitive vertebrates and in species closely related to vertebrates will offer valuable information to distinguish the two models.

It has been documented that the expression of the DAZ family genes well correlates with their specific roles in different stages of germline development. For example, the *Drosophila* *boule* is expressed in meiotic male germ cells and required for the meiotic progression in spermatogenesis but not oogenesis, as mutant male flies have infertility with their male germ cells arrested at the G2/M transition in meiosis I [12]. The nematode *boule* homolog is expressed in meiotic female germ cells, and its mutations completely abolish fertility in hermaphrodites due to arrest at meiotic prophase in oogenesis [15]. Targeted disruption of mouse *Dazla* completely abolishes gamete production in both sexes, with female germ cells being arrested at the prophase of meiosis I and male germ cells being affected at the proliferating stage [35]. It has recently reported in cell cultures that the human *Dazl* functions in primordial germ cell formation, whereas *Daz* and *Boule* promote later stages of meiosis and development of haploid gametes [36], in accordance with their stage-specific expression [1]. Comparative expression analyses of the DAZ family genes in lower and higher vertebrates will provide useful information on the evolution and function of molecular mechanisms underlying gametogenesis and fertility.

**Materials and Methods**

**Fish and Chemicals**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in Singapore and approved by this committee (Permit Number: 27/09). Rainbow trout females were purchased from a fish farmer in Wrzburg, Germany, and adult testis samples were kindly provided by Dr. Goro Yoshizaki (Japan).
and the Heilongjiang Fisheries Research Institute (Ha’erbin, China). Chemicals and enzymes were from Sigma and Promega, respectively, unless otherwise indicated.

Isolation of cDNA sequence

Total RNA was isolated from adult tissues by using the Trizol Reagent (Invitrogen). To eliminate genomic DNA contamination, RNA samples were treated with RNase-free DNase (Promega). cDNA was synthesized by using MMLV reverse transcriptase (Invitrogen) as described [11]. PCR was performed by using two primers each for boule (bolf, CGATGGAGAACGAAATAACCC; bolr, GAGAGCCAGGGCCCTGAGCGGGAGG), dazl (dazf, AT- TTATTATTCTGTTTTAACGGTTTTAGAA; dazr, AGAGGCT- CATGGCTCAGCAGA) and vasa (vasf, GGGACCTCATGGCC- GTGTGCC; vasr, ATCACTCCCATTCGTCGTCG; accession number, AB032566.1). As a control, b-actin was amplified from the same set of cDNA samples by using two primers TA1, TTCAACAGCCCTGCCATGTAC; TA2 (CCTCCAATCCAGACAGAGTATT) according to the trout actin cDNA sequence (accession number, AF157514.1). A PCR reaction in a 20 µl volume contained a cDNA aliquot equivalent to 20 ng of total RNA. PCR was for 35 cycles of 20 s at 94°C, 20 s at 58°C and 60 s at 72°C. The PCR products were separated on 1.2% agarose gels and documented with a bioimaging system (Synoptics).

Sequence Analysis

The PCR products of the 808-nt boule cDNA, 706-nt dazl cDNA and 1.2-kb vasa cDNA were cloned into pGEM-T vector, resulting in pOMbol, pOMdazl and pOMvasa. The inserts were sequenced in both directions on the Applied Biosystems 3130xl (Applied Biosystems, MA). BLAST searches were run against public databases by using BLASTN for nucleotide sequences and BLASTP for protein sequences. Multiple sequence alignment was conducted by using the Vector NTI suite 8. Phylogenetic trees were constructed by using the DNAMAN package.

Sections

The trout testis and ovary were fixed overnight or longer at 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. They were dehydrated by passing through an increasing series of ethanol (70%, 80%, 90% and 100%) for 1 hour each. After an additional treatment in 100% ethanol, the samples were treated for 30 min each in a mix of Histoclear:ethanol (1:1) and 100% Histoclear. After 30-min incubation in paraffin at 60°C, the samples were treated overnight in paraffin at 60°C and embedded in paraffin. The testis was cut at 5 µm and ovary at 8 µm on the Leica RM2135 Microtomes (Leica, Germany). Slides of sections were dewaxed by immersion in xylene with three changes, each for 10 min. After rehydration through a descending ethanol series (100%, 90%, 70%, 50% and 30%), the slides were subjected to treatments two times with PBS containing 0.1% Tween-20 (PBST) for 5 min each, once with 0.1N HCl for 5 min and three washes in PBST for 5 min each. The samples were treated with proteinase K (10 µg/ml) for 30 min at 37°C, washed three times in PBST for 5 min each, and refixed in 4% for 15 min at room temperature. After three PBST rinses, the samples were subjected to in situ hybridization.

Chromogenic in situ hybridization

Chromogenic ISH by using BCIP/NBT as substrates was performed as described previously with minor modifications [33]. Briefly, pOMbol, pOMdazl and pOMvasa were linearized with Xho I and Sac II and used for the synthesis of sense and anti-sense RNA probes from SP6 or T7 promoter by using the digoxigenin (DIG) Labeling Kit (Roche) for dazl and vasa, or the FITC RNA Labeling Kit (Roche) for boule. The RNA probes were treated with

Figure 9. Expression of boule and dazl transcripts in trout spermatogenesis. Adult testicular cryosections were hybridized to antisense RNA probes and the signals were visualized by fluorescence staining. Nuclei were stained blue by DAPI. (A) Bright field. (B) boule signal. (C) dazl signal. (D) Merge. Different stages of spermatogenesis are indicated: sg, spermatogonia; sc1, primary spermatocytes; sc2, secondary spermatocytes; st, spermatids; sm, sperm. The boule RNA forms the diffuse signal, whereas the dazl RNA forms speckles in spermatogonia and spermatocytes, and condenses in the chromatoid body (asterisks) of spermatids and sperm. Scale bars, 20 µm.
doi:10.1371/journal.pone.0015910.g009
RNase-free TURBO DNase (Ambion) and purified by using the LiCl method according to the supplier’s instruction (cat #AM1340, Ambion). DIG probe and FITC probes were denatured incubation at 90°C for 5 min followed by rapid cooling on ice. Chromogenic ISH was performed as described previously [33]. Briefly, the probe was added at ~1 μg/ml to the hybridization buffer (50% formamide, 5 × SSC, 500 μg/ml yeast tRNA, 0.1% Tween-20 and 50 μg/ml heparin). The samples on each slide were covered with 100 μl of the hybridization buffer containing a probe and wrapped in parafilm. The slides were put into a Petri dish layered at bottom a Whatman paper presoaked with 2 × SSC containing 0.1% Tween-20 (SSCT) and 50% formamide. The dish was sealed with parafilm and incubated overnight at 65°C. The slides were washed two times in 2 × SSCT-50% formamide for 30 min each, two times in 2 × SSCT and two times in 0.2 × SSCT for 30 min each. After incubation in blocking buffer (PBST containing 2% blocking reagent and 10% goat serum) for 1 hour at room temperature, the samples were incubated with either the alkaline phosphatase (AP) conjugated anti-DIG-antibody or AP-conjugated anti-FITC-antibody (Sigma).
at 1:2000 in the blocking buffer for 2 hours at room temperature. Following six PBST washes, and preincubation 2 times in the pre-staining buffer (NTMT; 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-Cl, pH 9.5, 0.1% Tween-20) for 15 min each, the samples were incubated in the staining buffer (0.1 mg/ml NBT/BCIP in NTMT) at room temperature for 30–60 min at 4°C. Color development was microscopically monitored at regular intervals. Nuclear staining was done by using 4’-6 Diamidino-2-phenylindole (DAPI; 1 μg/ml) and embedded in the Gold Antifade reagent (Invitrogen) for microscopy.

Fluorescence in situ hybridization

Two-color fluorescence in situ hybridization (FISH) was performed by using the tyramide signal amplification (TSA™ Plus Fluorescence Systems according to the manufacturer’s instruction [NEL756, PerkinElmer Life Science] [18]. Briefly, after hybridization and blocking as described above, the samples were incubated with the horseradish peroxidase (POD)-conjugated anti-FITC-antibody (Sigma) at a 1:100 dilution in the TSA Amplification buffer at room temperature to detect the FITC-labeled probes. Following six PBST washes the samples were incubated for 30 minutes in the TSA-Fluorescein at a 1:100 dilution in the TSA Amplification buffer for 1:400 dilution in the TSA Amplification buffer. The samples were then subjected to detection of the DIG-labeled probe: They were treated for 1 hour in blocking buffer with 1% H₂O₂ and incubated for two hours with the POD-conjugated anti-DIG antibody (Sigma) at a 1:2000 dilution, followed by incubation in TSA-Cy3 for 30 min. The samples were finally stained for nuclei by using DAPI and embedded in the Gold Antifade reagent (Invitrogen) for microscopy. Microscopy and Photography

Observation and photography on Leica MZ5III stereo microscope, Zeiss Axiovertinert and Axiovert upright microscopes with a Zeiss AxioCam M5Re digital camera (Zeiss Corp) were as described previously [11].

Acknowledgments

We thank J. Deng for collecting fish samples, Dr. Goro Yoshizaki, Japan, for providing trout testis samples. This work contains part of Qian Shen and Foong Mei Wong’s honor’s thesis.

Author Contributions

Conceived and designed the experiments: YH. Performed the experiments: ML QS HX FW JC ZL NH LW HZ BM. Analyzed the data: ML QS HX. Contributed reagents/materials/analysis tools: ZL NH HZ. Wrote the paper: YH.

References

1. Xu EY, Moore FL, Pera RA (2001) A gene family required for human germ cell development evolved from an ancient metazoic gene conserved in metazoans. Proc Natl Acad Sci U S A 98: 7414–7419.
2. Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, et al. (1995) Diverse spermatogonial defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 10: 303–309.
3. Saxena R, Brown LG, Hawkins T, Alagappan RK, Slateky H, et al. (1996) The DAZ gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. Nat Genet 14: 292–299.
4. Reijo R, Schmalian J, Dinulos MB, Jaffe T, Brown LG, et al. (1996) Mouse autosomal homolog of DAZ, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. Genomics 35: 346–352.
5. Sesboue E, Barbaux S, Bourgeron T, Nishi S, Aguilhak A, et al. (1997) Gene sequence, localization, and evolutionary conservation of DAZLA, a candidate male sterility gene. Genomics 41: 227–235.
6. Cooke HJ, Lee M, Kerr S, Ruggiu M (1996) A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads. Hum Mol Genet 5: 513–516.
7. Ellis S, Battellier F, Couty I, Balzergh S, Martin-Magniette ML, et al. (2000) Search for the genes involved in oocyte maturation and early embryo development in the hen. BMC Genomics 9: 110.
8. Houston DW, Zhang J, Maines JZ, Wasserman SA, King ML (1998) A Xenopus DAZ-like gene encodes an RNA component of germ plasm and is a functional homolog of Drosophila boule. Development 125: 171–180.
9. Johnson AD, Bachvarova RF, Drum M, Masi T (2001) Expression of axolotl DAZ-like gene encodes an RNA component of germ plasm and is a functional homolog of Drosophila boule. Development 125: 171–180.
10. Maegawa S, Yasuda K, Inoue K (1999) Maternal mRNA localization of the DAZL-related protein boule in Drosophila spermatogenesis. Dev Biol 204: 402–415.
11. Xu H, Li M, Li Z, Li M, Wang L, Hong Y (2009) Boule is present in fish and bisexualy expressed in adult and embryonic germ cells of medaka. PLoS One 4: e6097.
12. Cheng MH, Maines JZ, Wasserman SA (1998) Biphatic subcellular localization of the DAZL-related protein boule in Drosophila spermatogenesis. Dev Biol 204: 567–576.
13. Xu EY, Moore FL, Pera RA (2000) The Caenorhabditis elegans homolog of deleted in azoospermia is involved in the sperm/oocyte switch. Mol Biol Cell 11: 3147–3155.
14. Xu EY, Lee DF, Klebs A, Turek PJ, Kornberg TB, et al. (2003) Human BOULE gene rescues meiotic defects in infertile flies. Hum Mol Genet 12: 169–173.
15. Karashima T, Sugimoto A, Yamamoto M (2000) Caenorhabditis elegans homolog of the human azoospermia factor DAZ is required for oogenesis but not for spermatogenesis. Development 127: 1069–1079.
16. Margaiva S, Yasumita M, Yasuda K, Inoue K (2002) Zebrafish DAZ-like protein controls translation via the sequence ‘GUUC’. Genes Cells 7: 971–984.
17. Peng JX, Xie JL, Zhou L, Hong YH, Gai JF (2000) Evolutionary conservation of Dazl genomic organization and its continuous and dynamic distribution throughout germline development in eukaryotic gel carp. J Exp Zool B Mol Evol 302: 855–871.
18. Xu H, Li Z, Li M, Wang L, Hong Y (2009) Boule is present in fish and bisexually expressed in adult and embryonic germ cells of medaka. PLoS One 4: e6097.
19. Yamanoue Y, Miya M, Inoue JG, Matsura K, Nishida M (2006) The mitochondrial genome of spotted green pufferfish Tetraodon nigrivordalis (Teleostei: Tetraodontiformes) and divergence time estimation among model organisms in fishes. Genes Cells 11: 29–39.
20. Yoshizaki G, Nakatsukisa T, Tomimasa H, Takeuchi T (2000) Cloning and characterization of a vasa-like gene in rainbow trout and its expression in the germ cell lineage. Mol Reprod Dev 55: 364–371.
21. Takeuchi Y, Yoshizaki G, Sakatani S, Takeuchi T (2000) Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. Int J Dev Biol 44: 323–326.
22. Takeuchi Y, Yoshizaki G, Kobayashi T, Takeuchi T (2002) Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the vasa gene promoter. Biol Reprod 67: 1087–1092.
23. Takeuchi Y, Yoshizaki G (2004) Biotechnology: surrogate broodstock produces salmonids. Nature 430: 628–630.
24. Timmermans LP, Parmentier HK, van den Boogaart JG (1985) Surface markers of male germ cells in early development of carp (Cyprinus carpio L.) and the blood-testis barrier in fish. A study with monoclonal antibodies and horseradish peroxidase (HRP). Cell Biol Int Rep 9: 515.
25. Lin WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
26. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
27. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
28. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
29. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
30. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
31. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
32. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
33. Liu WS, Wang A, Uno Y, Galitza D, Beattie CW, et al. (2007) Genomic structure and transcript variants of the bovine DAZL gene. Cytogenet Genome Res 116: 65–71.
30. Knaut H, Pelegri F, Bohmann K, Schwarz H, Nusslein-Volhard C (2000) Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. J Cell Biol 149: 875–888.

31. Yoon C, Kawakami K, Hopkins N (1997) Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124: 3157–3165.

32. Shinomiya A, Tanaka M, Kobayashi T, Nagahama Y, Hamaguchi S (2000) The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, Oryzias latipes. Dev Growth Differ 42: 317–326.

33. Xu H, Gui J, Hong Y (2003) Differential expression of vasa RNA and protein during spermatogenesis and oogenesis in the gibel carp (Carassius auratus gibelio), a bisexually and gynogenetically reproducing vertebrate. Dev Dyn 230: 872–882.

34. Kito G, Aramaki S, Tanaka K, Soh T, Yamasuchi N, et al. Temporal and Spatial Differential Expression of Chicken Germline-specific Proteins cDAZL, CIDH and CVH During Gametogenesis. J Reprod Dev 56: 341–346.

35. Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, et al. (1997) The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389: 73–77.

36. Kee K, Angeles VT, Flores M, Nguyen HN, Reijo Pera RA (2009) Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature 462: 222–225.

37. Liu L, Hong N, Xu H, Li M, Yan Y, et al. (2009) Medaka dead end encodes a cytoplasmic protein and identifies embryonic and adult germ cells. Gene Expr Patterns 9: 541–548.