Cell Tissue Res
DOI 10.1007/s00441-014-2095-2

REGULAR ARTICLE

Differential expression of fertility genes *boule* and *dazl* in Chinese sturgeon (*Acipenser sinensis*), a basal fish

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Received: 24 June 2014 / Accepted: 8 December 2014

Abstract The gene family *DAZ* (deleted in Azoospermia), including *boule*, *dazl* and *DAZ*, performs highly conserved functions in germ cell development and fertility across animal phyla. Differential expression patterns have been demonstrated for the family members in invertebrates and vertebrates including fish. Here, we report the identification of *boule* and *dazl* and their expression at both RNA and protein levels in developing and mature gonads of Chinese sturgeon (*Acipenser sinensis*). Firstly, the isolation of the *boule* and *dazl* genes in Chinese sturgeon and the observation of the two genes in coelacanth suggest that *dazl* originated after the divergence of bony fish from cartilaginous fish but before the emergence of the Actinistia. Quantitative real-time PCR and western blot analyses reveal that *boule* and *dazl* RNA and proteins are restricted to the testis and ovary. In situ hybridization and fluorescent immunohistochemistry show that the bisexual mitotic and meiotic germ cell expression of *dazl* RNA and protein is conserved in vertebrates, while Chinese sturgeon *boule* RNA and protein exhibit mitotic and meiotic expression in the testis, and also likely display mitotic and meiotic expression in female. Moreover, we directly demonstrate for the first time that sturgeon Balbiani body/mitochondrial cloud disperses in the cytoplasm of early developing oocytes and co-localizes with Dazl to some extent. Finally, urbilaterian *boule* may also have an ancestral function in oogenesis. Taken together, these results provide useful information on the evolution of *DAZ* family genes, expression patterns and functions in animal reproduction.

Keywords *Boule* · *Dazl* · Germ cell · Gametogenesis · Chinese sturgeon

Introduction

The mechanisms of gametogenesis, oogenesis in the ovary and spermatogenesis in the testis, are complex and divergent among metazoans. The *DAZ* (deleted in Azoospermia) gene family has been suggested to play essential roles in the generation of male and female gametes in both vertebrates and invertebrates (Shah et al. 2010; Xu et al. 2001). This gene family consists in a Y-linked *DAZ* gene cluster and the autosomal *dazl* and *boule* genes, all of which encode germ cell-specific RNA-binding proteins with a common RNA-recognition motif and at least one copy of DAZ repeat (Xu et al. 2001). The Y-linked *DAZ* gene cluster is restricted to catarrhine primates (Agulnik et al. 1998; Gromoll et al. 1999), while *dazl* homologs are found only in vertebrates, such as zebrafish (Maegawa et al. 1999), medaka (Xu et al. 2007), rainbow trout (Li et al. 2011), gibel carp (Peng et al. 2009), *Xenopus* (Houston et al. 1998), axolotl (Johnson et al. 2001), chicken (Rengaraj et al. 2010), mouse (Cooke et al. 1996) and human (Seboun et al. 1997), where they are more closely

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Electronic supplementary material The online version of this article (doi:10.1007/s00441-014-2095-2) contains supplementary material, which is available to authorized users.

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Published online: 16 January 2015

related to *DAZ* than to *boule* (Xu et al. 2001). Consequently, the autosomal *dazl* gene is considered the ancestor of the *DAZ* gene through transposition and repeated amplification and pruning (Gromoll et al. 1999; Shan et al. 1996). However, the third family member, *boule*, has been identified in species ranging from sea anemones through humans (Shah et al. 2010). It is reasonable, therefore, to propose that the *dazl* gene arose from a duplication of *boule* during vertebrate evolution (Shah et al. 2010; Xu et al. 2001).

The *DAZ* family genes examined so far perform a conserved function in germ cell development, which have been demonstrated by rescue experiments. Human *BOULE* can rescue partial testicular defects of fruit flies with *boule* mutant (Xu et al. 2003). Furthermore, frog *dazl* can restore fly *boule* mutant to some extent, though frog *dazl* show a germline function different from fly *boule* (Houston et al. 1998; Houston and King 2000). Additionally, both human *DAZ* and *DAZL* are able to partially rescue germ cell numbers of *Dazl* null mouse (Slee et al. 1999; Vogel et al. 2002). Taken together, the homologs of *DAZ* family genes from different species share some functional conservation but different homologs can also partially replace each other.

Members of the *DAZ* gene family, however, present a striking difference in sex- and stage-specific expression, albeit they are restricted to germ cells in nearly all animals. Human *DAZ* and its protein are exclusively expressed in the male gonad, especially in the mitosis stage (Huang et al. 2008; Saxena et al. 1996). However, *dazl* homologs are present in both male and female germlines. Generally, they are transcribed continuously from embryonic stem cells to primordial germ cell specification and into spermatogenesis up until round spermatids in males (Vangompel and Xu 2011) and in all the stages of oogenesis (Li et al. 2011). Such a protein expression pattern has been identified in frogs (Mita and Yamashita 2000), mice (Ruggiu et al. 1997) and humans (Reijo et al. 2000). Nonetheless, the post-meiotic DAZL protein expression in mammals is disputed (Reijo et al. 2000; Vangompel and Xu 2011; Xu et al. 2001). Finally, the expression pattern of the third family member *boule* varies according to species. The homologs of *boule* are mainly expressed in the testes of flies (Eberhart et al. 1996), sea urchins and mice (Shah et al. 2010), while their transcripts expression has also been reported in the both testes and ovaries of *Macrostomum lignano* (Kuales et al. 2011), medaka (Xu et al. 2009) and rainbow trout (Li et al. 2011) and only in the ovaries of *C. elegans* (Karashima et al. 2000). Additionally, they are predominantly in the meiosis stage. In a word, the sex- and stage-specificity of *DAZ* family genes varies considerably depending on the family member and species.

Sturgeons are Acipenseriformes and therefore among the most primitive Actinopterygii. Previous studies have suggested that *dazl* most likely originated in the ancestral lineage of bony fish (Shah et al. 2010). Thus, we chose the Chinese sturgeon (*Acipenser sinensis*) to explore the origin and evolution of *DAZ* family genes in vertebrates. On the other hand, Chinese sturgeon is a rare and endangered species, due to anthropogenic interference, such as over-fishing, damming of rivers and pollution (Birstein et al. 1997; Wei et al. 1997). Controlled propagation has been successfully conducted (Wei et al. 2013) and proven to be important for its conservation. However, Chinese sturgeon is an extremely late and asynchronous maturing species (8–18 years for males and 14–26 years for females) (Chen et al. 2006; Wei et al. 1997), making culture and conservation costly and time consuming. In recent years, germ cell transplantation has been successfully conducted in some fish species (Lacerda et al. 2013) and is believed to be an available and rapid method for protecting endangered fishes. Germ cells isolation is the first step in implementation of this plan. The *DAZ* family genes can be used as germ cells markers in the species examined so far (Shah et al. 2010; Vangompel and Xu 2011). In this study, we identify *boule* and *dazl* in Chinese sturgeon, examine their mRNA and protein expression patterns and observe their cellular and subcellular localization in the gonad.

Materials and methods

Animals and samples

Chinese sturgeons used in this study were cultured in Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Deep anaesthesia was induced by a 0.05 % solution of 3-aminobenzoic acid ethyl ester methanesulfonate-222 (MS-222) (Sigma, USA). Tissue samples from seven 2.5-year-old Chinese sturgeons (approximately 1.23 m in length and 7.3 kg in weight) were collected within 30 min of exsanguinations by tailing and immediately dipped into liquid nitrogen and stored at −80 °C. Moreover, small pieces of testis were removed surgically from 12-year-old males (approximately 1.86 m in length and 49.6 kg in weight). Additionally, zebrafish females were kindly provided by Dr. Jian-Fang Gui. The experimental procedures were based on the standards of the Chinese Council on Animal Care.

Isolation of cDNA sequence

Total RNA was isolated from different tissues by using the RNeasy Plus Mini Kit (Qiagen). The RNA quality and purity were measured by Nanodrop 2000. SMART cDNA was synthesized by using the SMARTer™ RACE cDNA Amplification Kit according to the manufacturers' instructions (Clontech). To obtain fragments of the Chinese sturgeon *boule* and *dazl* cDNA, RT-PCR was performed with degenerate primers (B-F and B-R, D-F and D-R) designed by conserved amino acid sequences (Table 1). After sequencing, the cloned

Table 1 Primers used for all the experiments

Primer name	Sequence (5′–3′)	Usage
B-F	5′> ATCCCYAAYCGSMTYTTTGT <3′	Amplification of fragments of *boule* and *dazl*
B-R	5′> AAGTARGCNACDCCRTTGTG <3′	
D-F	5′> TTYGTNGGHGGHATHGAYATG <3′	
D-R	5′> RTAGYTCAYKGGCAYCTGTG <3′	
B-5′-R1	5′> CTGAGAAAGGGAATCCAGCATAG <3′	5′ RACE
B-5′-R2	5′> TCTTCTGCGCATCGTCCTGTGTC <3′	
D-5′-R1	5′> GCTGCCTAACATTTTCCATCATT <3′	
D-5′-R2	5′> AAGGAGCAGTCATCCATTGTGTAG <3′	
B-3′-F1	5′> TGTGGGGGGCATCGACTTCA <3′	3′ RACE
B-3′-F2	5′> CCTATGCTGGATTCCCTTTCTCA <3′	
D-3′-F1	5′> GATTTGGGGCAGTGAAGGAAGT <3′	
D-3′-F2	5′> GGACCTACACAATGGATGAC <3′	
B-RT-F	5′> GGGTGGTGAACGAGGTGAAGATA <3′	Real-time PCR
B-RT-R	5′> CCACTTGCTGTTTGCGGATG <3′	
D-RT-F	5′> GATTTGGGGCAGTGAAGGAAGT <3′	
DRT-R	5′> TGGCTGCAACACACACAATACAC <3′	
actin-RT-F	5′> CCTTCTTGGGTATGGAATCTTGC <3′	
actin-RT-R	5′> CAGAGTATTTACGCTCAGGTGGG <3′	
B-s-T7-F	5′> TAATACGACTCACTATAGGGTACCAGGCTCCTACCCAGTGTC <3′	*boule* and *dazl* in situ probes amplification
B-s-R	5′> TCAAATGCTGCCACCTTGTCAG <3′	
B-anti-F	5′> TACCAGGCTCCTACCCAGTGTC <3′	
B-anti-T7-R	5′> TAATACGACTCACTATAGGGTCAAATGCTGCCACCTTGTCAG <3′	
D-s-T7-F	5′> TAATACGACTCACTATAGGGCTTGGACCCGCAATAATGAAAGA <3′	
D-s-R	5′> CACACATGGCAAGCAGCCTTTAG <3′	
D-anti-F	5′> CTTGGACCCGCAATAATGAAAGA <3′	
D-anti-T7-R	5′> TAATACGACTCACTATAGGGCACACATGGCAAGCAGCCTTTAG <3′	
B-E-F	5′> GAATTCCAGCAAGTGGGGATCC <3′	Plasmids construction for recombinant protein expression
B-E-R	5′> CTCGAGCTAGTTGAGAGAGTCAGTGC <3′	
D-E-F	5′> GGATCCATGTCTGTCAAAGAATCACAG <3′	
D-E-R	5′> CTCGAGTCATAACAGGGTCAGCAGT <3′	
UPM	5′> CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAG <3′ and 5′> CTAATACGACTCACTATAGGGC <3′	RACE
NUP	5′>AAGCAGTGGTATCAACGCAGAGT <3′	

fragments were used to design gene-specific primers for 5′ and 3′ RACEs. Then, the full-length cDNA of *boule* and *dazl* were amplified by nest PCR. For the 5′ end cDNA of *boule* and *dazl*, B-5′-R1/D-5′-R1 and anchor primer UPM (Table 1) were used for the first round of PCR amplification. The first round PCR product was then used as the template for the second round of PCR with primers B-5′-R2/D-5′-R2 and primer NUP (Table 1) to obtain the 5′ end cDNA. Similarly, for the 3′ end cDNAs of *boule* and *dazl*, B-3′-F1/D-3′-F1 and anchor primer UPM (Table 1) were used for the first round of PCR amplification. The first round PCR product was then used as the template for the second round of PCR with primers B-3′-F2/D-3′-F2 and primer NUP to obtain the 3′ end cDNA. The PCR products were visualized by electrophoresis of ethidium bromide stained agarose gel, cloned into pMD19-T vector (Takara) and sequenced.

Sequence analysis

The cDNA and deduced amino acid sequences were analyzed by the BLAST program (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed with the CLUSTAL X program (version 2.1), with the printing output shaded by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). In addition, the phylogenetic relationship of *DAZ* family genes was examined using the Bayesian inference. Bayesian analysis of phylogeny was performed with MrBayes 3.1 (http://mrbayes.sourceforge.net). Trees were constructed using a HKY+I+G model and four Markov chains were run in parallel for 2,000,000 generations, sampling every 100th generation. Graphical representation of the phylogenetic tree was obtained with FigTree software (http://tree.bio.ed.ac.uk/software/figtree).

Quantitative real-time PCR

For quantitative real-time PCR, total RNA extracted from different tissues (including liver, spleen, heart, ovary, testis, kidney, intestine and brain) from 2.5-year-old cultured Chinese sturgeons, was isolated by using the RNeasy Plus Mini Kit (Qiagen). Total RNA from each sample was reverse transcribed with the PrimeScript RT reagent Kit With gDNA Eraser (Takara) as described (Ye et al. 2012). Temporal tissue expression of *boule* and *dazl* mRNA was analyzed using quantitative real-time PCR performed in a DNA Engine Chromo 4 real-time system (BioRad). Reactions were carried out at a final volume of 20 μl, containing 1 μl DNA sample, 10 μl 2×SYBR green real-time PCR master mix (BioRad), 0.2 μl of each primer and 8.6 μl H2O. The primer pairs used were B-RT-F and B-RT-R for *boule* (Table 1) and D-RT-F and D-RT-R (Table 1) for *dazl* with *β-actin* as an internal control. The amplification protocol was as follows: 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C and 15 s at 72 °C. Negative controls were prepared with sterile deionized water instead of cDNA template. All samples were analyzed in triplicate and the relative expression level of target genes was calculated with the $2^{-\Delta CT}$ method (Schmittgen and Livak 2008).

In situ hybridization

Using T7 polymerase by in vitro transcription (DIG RNA labeling kit' Roche Molecular Biochemicals), sense and antisense probes for *boule* and *dazl* were generated directly from the PCR product that included T7 RNA polymerase binding sequence at the 5′ end of the forward and backward primer (Table 1), respectively. In situ hybridization was performed according to the published protocol with minor modifications (Thisse and Thisse 2008).

Antibody production

Two cDNA fragments encoding 230 amino acids (aa) of Boule (B-E-F and B-E-R; Table 1) and 224 aa of Dazl (D-E-F and D-E-R; Table 1) were subcloned into pGEX-4 t-1 expression vector. The recombinant fusion proteins were expressed in *E. coli* BL21 (DE3) and detected only in the inclusion body. The proteins were prepared and used to immunize mouse and rabbit, respectively, as previously described (Xia et al. 2007). Briefly, a mixture of equal volumes of complete Freund Adjuvant and purified protein (50 μg) was used for initial inoculation of mice. After 1 week, a mixture of equal volumes of incomplete Freund Adjuvant and purified protein (50 μg) was injected, with injections repeated four times at weekly intervals. Lastly, the mice were bled and serum samples were stored at −80 °C until use. Moreover, 500 μg of the purified protein with the gel was injected into the swollen lymphoid node of the white rabbits. Two booster injections of the same amount of antigen were administered in each of the following 2 weeks. Ten days following the final booster, blood samples were taken and serum samples were stored at −80 °C until use.

Western blot

Homogenates of somatic tissues of 2.5-year old Chinese sturgeon were prepared, dissolved in 12 % SDS-PAGE and blotted (Ye et al. 2012). The blot was stained with anti-Boule or anti-Dazl antibody and detected by alkaline phosphatase staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) as the chromogenic substrate (Ye et al. 2012).

Fluorescent immunohistochemistry

Chinese sturgeon gonad tissue was immediately immersed into 4 % paraformaldehyde for 12 h at 4 °C, dehydrated in a graded methanol series and frozen at −20 °C. Prior to analysis, samples were hydrated in a graded methanol series and immersed in phosphate buffer solution (PBS) buffer (containing 30 % saccharose) for 12 h at 4 °C. Then, samples were embedded in Optimal Cutting Temperature (O.C.T.; Sakure) and quickly cooled in liquid nitrogen. The cooled samples were cut at 8 μm with frozen microtomy (Leica, Germany) and collected on TESPA-coated slides. Subsequently, the fluorescent immunochemistry was conducted with both anti-Boule antibody (1:100 dilution) and anti-Dazl antibody (1:150 dilution). Fluorescent secondary antibodies against mouse and rabbit used for detection were TRITC (1:150 dilution) and FITC (1:200 dilution), respectively.

Mitochondrial staining

The sections were stained for mitochondria detection with the MitoTracker reagent (Invitrogen) at 100 nM according to the supplier's instructions and 4′-6-Diamidino-2-phenylindole (DAPI) staining for nuclei before visualization.

Microscopy and photography

Cross-sections after RNA in situ hybridization were observed and photographed under an Olympus upright microscope (BX51). Slides after fluorescent immunohistochemical staining were examined by a Confocal Microscope (Zeiss).

Statistical analysis

Data were presented as mean ± SD. The data were assessed by the Student's *t* test.

Results

Gene identification

Using degenerate primers and RACE strategy, we cloned the full-length cDNA sequences of Chinese sturgeon *boule* and *dazl*. The complete cDNA sequence of *boule* (accession no. KF850542) was 2557 base pairs (bp) in length, contained a 139-bp 5′-untranslated region (UTR) and a 1065-bp open reading frame for 354 amino acid residues (Fig. 1a). The predicted Boule shared 31 and 64 % identity with medaka and human BOULE proteins. On the other hand, the *dazl* cDNA (accession no. KF850543) was 2337 bp in total length and consisted in a 126-bp 5′-UTR and a 675-bp coding sequence for 224 aa (Fig. 1b). The deduced amino acid sequences of Dazl were 62 and 54 % identical to trout and medaka Dazl, respectively and about 40 % identical to mammalian DAZL.

The conserved region of Boule and Dazl resided within the RNA recognition motif (RRM). Multiple amino acid sequence alignments of the RRM revealed the most conserved residues to be in the two RNP motifs, RNP1 and RNP2 (Fig. 2a, b). Moreover, phylogenetic analysis of the *DAZ* family placed the origin of the *Boule* gene prior to the divergence of Bilateria from Cnidaria and showed Boule proteins were present in both invertebrates and vertebrates, whereas Dazl and DAZ were found in Osteichthyes and catarrhine primates, respectively, clustered together in a clade (Fig. 3). This supported the widespread ancient occurrence of Boule and the late arrival of Dazl in vertebrate evolution and DAZ in primate evolution.

The definite time of the first *boule* gene duplication leading to *dazl* remains unclear, although *dazl* originated around the time of vertebrate radiation (Shah et al. 2010). The *boule* (accession no. ENSLACG00000015867) and *dazl* (accession no. ENSLACG00000002895) were both present in coelacanth *Latimeria chalumnae* (Amemiya et al. 2013) and perfectly matched Boule and Dazl proteins consensus sequences, respectively (Shah et al. 2010). Furthermore, alignments of the deduced amino acid sequence of the Boule and Dazl RRM motif (Fig. 2a and b) and the molecular phylogenetic tree of the *DAZ* family (Fig. 3), specified coelacanth *boule* and *dazl* genes as true *boule* and *dazl* orthologs. This, along with the fact that a *dazl* ortholog is not found in cartilaginous fish, jawless fish, Tunicata, Cephalochordata, Echinodermata and Hemichordata (Shah et al. 2010), strongly suggests that the gene duplication resulting in *boule* and *dazl* occurred in the ancestral Actinistia but after the divergence of Osteichthyes from cartilaginous fish.

Gonad-specific expression of RNA and protein

The tissue distribution of *boule* and *dazl* was examined by quantitative real-time PCR. As shown in Fig. 4a, the transcripts of *boule* and *dazl* were detected in ovary and testis but absent in any of the somatic organs examined (liver, spleen, heart, kidney, intestine and brain). The expression of *boule* in testis was 17.94 times that in ovary, while *dazl* expression in testis was 0.53 times that in ovary. On the other hand, in ovary, the *dazl* transcripts level was 21.48 times that in *boule*, while the *boule* expression in testis was 1.58 times that in *dazl*.

The presence of Boule in ovary of vertebrates has not been demonstrated at the protein level, although ovarian expression of *boule* mRNA was reported in medaka (Xu et al. 2009), trout (Li et al. 2011) and mouse (Shah et al. 2010). Consequently, we prepared two recombinant proteins containing 230 amino acids of Boule and 224 amino acids of Dazl and obtained a mouse anti-Chinese sturgeon Boule antibody and a rabbit anti-Chinese sturgeon Dazl antibody, respectively. Subsequently, the specificity of the two polyclonal antibodies was confirmed (Supplementary Fig. 1). As shown in Fig. 4b, internal control β-Actins were found in all tissues examined, while a single band of the expected size (approximately 39 kDa for Boule and 25 kDa for Dazl) was detected exclusively in male and female gonads but not in somatic tissues by western blot, in accordance with their mRNA expression characteristics.

Differential germ cell-specific expression of RNA in the ovary

To investigate the cellular distribution of *boule* and *dazl* transcripts in ovary, in situ hybridization was employed. Chen et al. (2006) reported that, in terms of histology, sex differentiation of the gonads of Chinese sturgeon is completed by the age of 9 months, at which time the gonads enter stage I, with the mitosis of spermatogonia and oogonia. When sturgeons are 2.5–3.0 years old, the ovaries reach stage II, with the appearance of primary oocytes. Hence, the ovaries of Chinese sturgeon examined in this study had not reached meiosis, at which point primary oocytes form and grow continuously. As shown in Fig. 5a, d, the *boule* and *dazl* RNA was found only in germ cells but barely in surrounding somatic cells, while no reproducible signals could be detected by the sense probe (Supplementary Fig. 2). In detail, a weak but detectable signal for *boule* and *dazl* was observed in oogonia and remarkably increased in primary oocytes, which were dispersed in the ooplasm.

As described previously (Chang et al. 2004; Kosaka et al. 2007; Li et al. 2011; Peng et al. 2009; Xu et al. 2009), *dazl* co-localized with a cytoplasm structure, named the Balbiani body (BB) or mitochondrial cloud (MC) (King et al. 2005), containing mitochondria, endoplasmic reticulum and germ plasm RNAs. In particular, this structure could be detected by a mitochondrial marker, such as MitoTracker. In order to ascertain whether Chinese sturgeon *dazl* was concentrated in the MC, the oocytes were stained using MitoTracker, with zebrafish oocytes as positive control. Surprisingly, the MC diffused in the ooplasm of Chinese sturgeon (Supplementary Fig. 3d–f),

a


1 GAGCGAAGGGAGGCCATTGAAGACAGTGTTGTATCGACCCGGT
44 ACAGGAGAACTTCAGTTCATTATCACTTATTATAATATTTAAATATATATTTTTACATATTACTACTCATAGTTAGGCACCCCATTTATCGCTGCA
140 ATGATGGAAAACGAAATCACACAGACGAGCAGTCAGACCCAGACTGGCTCGCTGTCCCCCCCGAACGGCATGGCCCCCAGCCCCGGGAACCACGGA
1 M M E N E I T Q T S S Q T Q T G S L S P P N G M A P S P G N H G
236 AACCCCACCAGCGCCCCCCGCTATGGCACTGTCATCCCCAACCGCATCTTCGTGGGGGGCATCGACTTCAAAACCAATGAAAACGACCTGCGCAAG
33 N P T S A P R Y G T V I P N R I F V G G I D F K T N E N D L R K
332 TTCTTCTCTCACTACGGGGTGGTGAACGAGGTGAAGATAGTCAACGACAGGGCCGGAGTCTCCAAAGGGTATGGATTTGTGACGTTTGAGACACAG
65 F F S H Y G V V N E V K I V N D R A G V S K G Y G F V T F E T Q
428 GACGATGCGCAGAAGATACTGCAGGAGGCTGATAAACTCTTCTTCAGAGATAAAAAGCTGAATGTCGGCCCGGCCATCCGCAAACAGCAAGTGGGG
97 D D A Q K I L Q E A D K L F F R D K K L N V G P A I R K Q Q V G
524 ATCCCCTATGCTGGATTCCCTTTCTCAGGCTCGAGCCCCAGTGTGACCCCCCCTTCCCCGGCCACCATGTACTTCACCACCTCCACAGGGTACCCC
129 I P Y A G F P F S G S S P S V T P P S P A T M Y F T T S T G Y P
620 TACACCTACCACAACGGAGTGGCATACTTCCATGCCCCTGAAATCACCTCCGTGCCACAGCACTGGCCTCCCTGCTCCATCTCCAGCTCCCCAGTC
161 Y T Y H N G V A Y F H A P E I T S V P Q H W P P C S I S S S P V
716 ATGGTCACACACGAGGCTCATCCAGTATTACAGCAGCCAGCTTGTCATTACCAGGCTCCTACCCAGTGTCTTCCAGGCCAGTGGCAGTGGAGTCTC
193 M V T H E A H P V L Q Q P A C H Y Q A P T Q C L P G Q W Q W S L
812 CCGCAGTCCCCAGCCCCGGCCATGCCTCTTCTCTACATGCAGGCCTCTGATATCCTGTACCAGACCCAGGAGCTGCCGCAGGACGGTGGCTGTGCC
225 P Q S P A P A M P L L Y M Q A S D I L Y Q T Q E L P Q D G G C A
908 CCCTCCACCCTGCCCATGCTGGAGGCCACGGTCCCTGAGGCGTTCCCGGAGCACGGTGTGCAGCCAGCGTACCACCAGGTGTTCACACAGAGCCCC
257 P S T L P M L E A T V P E A F P E H G V Q P A Y H Q V F T Q S P
1004 GCCTGTATGCCGCAACTACCCATCCTGCAGCAGGAACCAGTGAAGGATCAGAGGCTGCACGCTATGCACAGGGGGTTCTCACAGTCTCCCATGGGG
289 A C M P Q L P I L Q Q E P V K D Q R L H A M H R G F S Q S P M G
1100 CTGACGCCCCGCTACACCCGCAGCTCACTCTACCACAGCCGCAAAGACTACCGACCCGAAGAGTCCATCCTTGCCCCCCCAGCCAGCACTGAGTCT
321 L T P R Y T R S S L Y H S R K D Y R P E E S I L A P P A S T E S
1196 CTCAACTAGAGCGGGAGAGAGGAGGAACCAGGGTCTCCGAGCTGCTCCTTCACCACACACACACTGGGGTTCCACTGGTCAGGGGGTCCGTTAGGG
353 L N *
1292 GGGCTCAGACACTGCAGAGAAGGAGGCACAGAAAAGCAGGTGTAGGTAGCAAATCTCGTCACTCTGTTTTGTTAAAGGCTGACGGGGTGATTTCAG
1388 TAAAACCTATATATAAAGGCACCTGCTATTAGCAGCTGCAAAATCCGAGTCTATGCATGAGTGACAGAGGCATGCTCTTTAATAAACTGTTAAAGT
1484 GGCCTGTACCGGTAGCTAATCACTCCCGTTGGTGATTGTTAAGTCATGTTTCATTGTTTTCATGAAAATGTGCTAGAAACCAACAACAAAATTTGT
1580 GCTTATGAAACTTTATTAAAATCATCAGAACAGTGCTCTGTTAAAGGCAGATTAGCTGCACTGTACAACTAGAATAAGCTCCAAATTCAGAGCACT
1676 GCAATAGCCACAGTTTAACCTGTTTGACTGTACTGCTTTCTTCGTACTCATTTACAGCATTTCCACTGCAGTCATCTTGTTTCACACGGCAGAATC
1772 TGTAGTTCAATTCTCTGCATTTTCCCTTTCAGAAAAAATTGTGCTCATGACCAGTTGGAGAAATTAGCTTTTGAGAATGTAACTCTTAAAGAGCCA
1868 GTATGACATTCACATAGGTTATAACAGTACTTGTAAGATGATCCTGGATCACGACAGTAGCCGCTGTGGTTGTAAGCGGTGGTCGGAACCACAGTG
1964 TTGGGTTGTCATCTCGTGCCCTGGCCCTAACGGATCATTCCAAAATAATAGAAAAAAAAGATTTTTAAAAAATCAACCAACTTTTTCTATATATTG
2060 TGGCAGCAAGAAATAAAACAGAACAGCATTTAACTCTTCTTTTTTTCTTTAAAAAGAAAAAGTGTATTATGATCATGGCTGCAACATTATAATAAC
2156 TGTGTGTGTAGACTAGTATGTTTTATATTGCTAGATGTATCCTTTTATTCTTGACTGGTGAAGACCATGGGTCTTGAAAACACACCTCTTAATTCA
2252 TGCATTGCAAATGTTTATACCAAACATGATTAAAAGATTGTAACCCAAGTGTCGTTCTCCTCCACTGACAAGGTGGCAGCATTTGAATGGCCGGTT
2348 ACTTCTATTGTGGTAAGCAGGTCAGTCTTCGGTCCTGCAGTCATTAGTTGTAGATGTAAAACAAGTGTGTGCTATTTCAAGTAGCACGTTGATGAC
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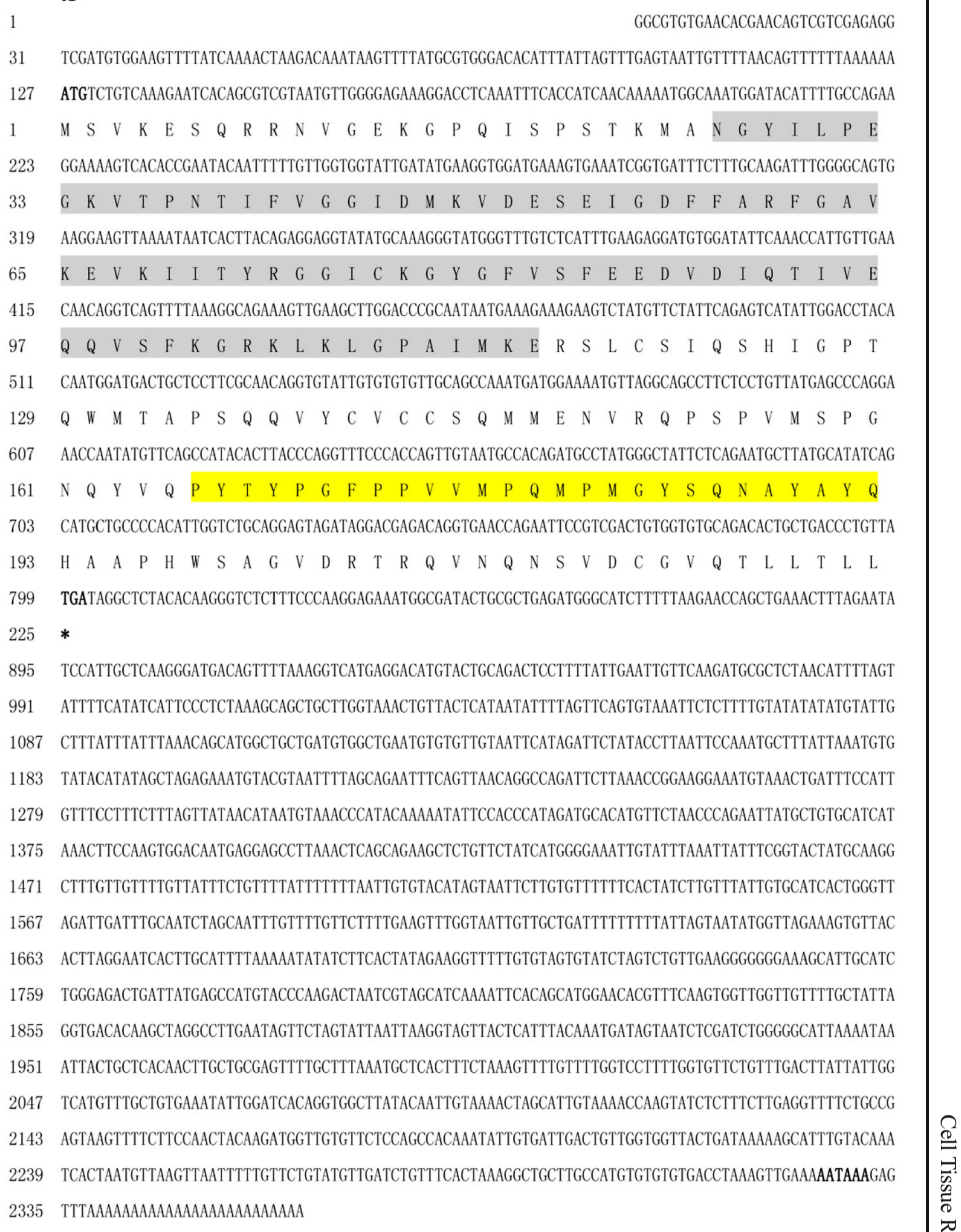
b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1 M S V K E S Q R R N V G E K G P Q I S P S T K M A N G Y I L P E
223 GGAAAAGTCACACCGAATACAATTTTTGTTGGTGGTATTGATATGAAGGTGGATGAAAGTGAAATCGGTGATTTCTTTGCAAGATTTGGGGCAGTG
33 G K V T P N T I F V G G I D M K V D E S E I G D F F A R F G A V
319 AAGGAAGTTAAAATAATCACTTACAGAGGAGGTATATGCAAAGGGTATGGGTTTGTCTCATTTGAAGAGGATGTGGATATTCAAACCATTGTTGAA
65 K E V K I I T Y R G G I C K G Y G F V S F E E D V D I Q T I V E
415 CAACAGGTCAGTTTTAAAGGCAGAAAGTTGAAGCTTGGACCCGCAATAATGAAAGAAAGAAGTCTATGTTCTATTCAGAGTCATATTGGACCTACA
97 Q Q V S F K G R K L K L G P A I M K E R S L C S I Q S H I G P T
511 CAATGGATGACTGCTCCTTCGCAACAGGTGTATTGTGTGTGTTGCAGCCAAATGATGGAAAATGTTAGGCAGCCTTCTCCTGTTATGAGCCCAGGA
129 Q W M T A P S Q Q V Y C V C C S Q M M E N V R Q P S P V M S P G
607 AACCAATATGTTCAGCCATACACTTACCCAGGTTTCCCACCAGTTGTAATGCCACAGATGCCTATGGGCTATTCTCAGAATGCTTATGCATATCAG
161 N Q Y V Q P Y T Y P G F P P V V M P Q M P M G Y S Q N A Y A Y Q
703 CATGCTGCCCCACATTGGTCTGCAGGAGTAGATAGGACGAGACAGGTGAACCAGAATTCCGTCGACTGTGGTGTGCAGACACTGCTGACCCTGTTA
193 H A A P H W S A G V D R T R Q V N Q N S V D C G V Q T L L T L L
799 TGATAGGCTCTACACAAGGGTCTCTTTCCCAAGGAGAAATGGCGATACTGCGCTGAGATGGGCATCTTTTTAAGAACCAGCTGAAACTTTAGAATA
225 *
895 TCCATTGCTCAAGGGATGACAGTTTTAAAGGTCATGAGGACATGTACTGCAGACTCCTTTTATTGAATTGTTCAAGATGCGCTCTAACATTTTAGT
991 ATTTTCATATCATTCCCTCTAAAGCAGCTGCTTGGTAAACTGTTACTCATAATATTTTAGTTCAGTGTAAATTCTCTTTTGTATATATATGTATTG
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1375 AAACTTCCAAGTGGACAATGAGGAGCCTTAAACTCAGCAGAAGCTCTGTTCTATCATGGGGAAATTGTATTTAAATTATTTCGGTACTATGCAAGG
1471 CTTTGTTGTTTTGTTATTTCTGTTTTATTTTTTTAATTGTGTACATAGTAATTCTTGTGTTTTTTCACTATCTTGTTTATTGTGCATCACTGGGTT
1567 AGATTGATTTGCAATCTAGCAATTTGTTTTGTTCTTTTGAAGTTTGGTAATTGTTGCTGATTTTTTTTTTATTAGTAATATGGTTAGAAAGTGTTAC
1663 ACTTAGGAATCACTTGCATTTTAAAAATATATCTTCACTATAGAAGGTTTTTGTGTAGTGTATCTAGTCTGTTGAAGGGGGGGAAAGCATTGCATC
1759 TGGGAGACTGATTATGAGCCATGTACCCAAGACTAATCGTAGCATCAAAATTCACAGCATGGAACACGTTTCAAGTGGTTGGTTGTTTTGCTATTA
1855 GGTGACACAAGCTAGGCCTTGAATAGTTCTAGTATTAATTAAGGTAGTTACTCATTTACAAATGATAGTAATCTCGATCTGGGGGCATTAAAATAA
1951 ATTACTGCTCACAACTTGCTGCGAGTTTTGCTTTAAATGCTCACTTTCTAAAGTTTTGTTTTGGTCCTTTTGGTGTTCTGTTTGACTTATTATTGG
2047 TCATGTTTGCTGTGAAATATTGGATCACAGGTGGCTTATACAATTGTAAAACTAGCATTGTAAAACCAAGTATCTCTTTCTTGAGGTTTTCTGCCG
2143 AGTAAGTTTTCTTCCAACTACAAGATGGTTGTGTTCTCCAGCCACAAATATTGTGATTGACTGTTGGTGGTTACTGATAAAAAGCATTTGTACAAA
2239 TCACTAATGTTAAGTTAATTTTTGTTCTGTATGTTGATCTGTTTCACTAAAGGCTGCTTGCCATGTGTGTGTGACCTAAAGTTGAAAAATAAAGAG
2335 TTTAAAAAAAAAAAAAAAAAAAAAAAAA

◀ **Fig. 1** Nucleotide and deduced amino acid sequences of Chinese sturgeon *boule* (**a**) and *dazl* (**b**). The nucleotide and amino acid residue number are shown on the *left*. The initiation codon, termination codon and consensus polyadenylation signal AATAAA are in *bold*. The RRM motif and DAZ motif are highlighted in *light gray* and *yellow*, respectively. The two sequences have been deposited in the GenBank nucleotide database, under accession no. KF850542 for *boule* and KF850543 for *dazl*

while zebrafish MC formed a spherical structure (Supplementary Fig. 3a–c). Nonetheless, the distribution of *dazl* RNAs seemed to overlap with the BB/MC.

Differential germ cell-specific expression of RNA during spermatogenesis

We then examined the expression of *boule* and *dazl* in the adult testis. The *boule* and *dazl* transcripts were restricted to germ cells but absent in surrounding somatic cells (Fig. 5b, e). The signal for *boule* peaked in spermatogonia, declined in primary and secondary spermatocytes and was faint in spermatid (Fig. 5c). Moreover, the *dazl* RNA was visible in spermatogonia and primary spermatocytes, slightly increased in secondary spermatocytes and detectable in spermatid (Fig. 5f).

Differential germ cell-specific expression of protein in the ovary

Up to now, there was a lack of knowledge on the protein expression and cellular distribution of Boule and Dazl in vertebrates. As a result, fluorescent immunohistochemistry was introduced to determine Chinese sturgeon Boule and Dazl proteins expression and subcellular localization. In the ovary of 2.5-year-old Chinese sturgeons, Boule and Dazl were detected exclusively in the cytoplasm of germ cells (Fig. 6a–d). Notably, both Boule and Dazl signals were strong in oogonia and remained high in primary oocytes. The pre-immune serums did not generate reproducible staining (Supplementary Fig. 4).

Differential germ cell-specific expression of protein during spermatogenesis

We furthered investigated the expression and subcellular localization of Boule and Dazl in adult testis. The two proteins were not found in any somatic cells but restricted to germ cells, in which they were cytosolic (Fig. 6e–h). Boule was abundant in spermatogonia and spermatocytes, reduced in spermatid and

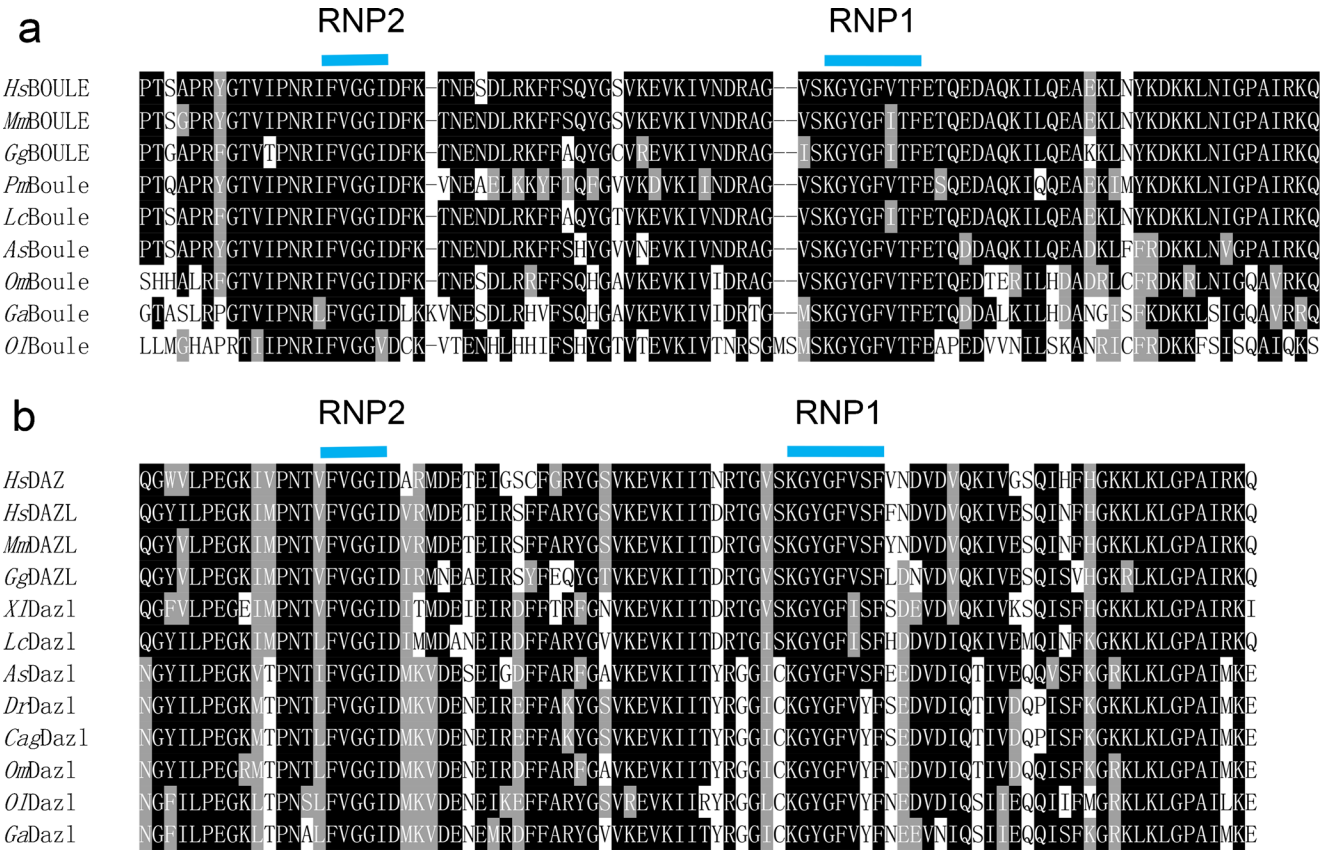

Fig. 2 Multiple amino acids sequence alignments of the RRM domain of Boule (**a**) and Dazl (**b**). Multiple alignments were performed with ClustalX 2.0 and refined using BOXSHADE 3.21. Identical residues and conservative substitutions are in *black* and *gray*, respectively. The *upper blue lines* indicate the most conserved motifs of Boule and Dazl RRM domain, RNP1 and RNP2. Species: *Hs*, *Homo sapiens*; *Bs*, *Bos taurus*; *Mm*, *Mus musculus*; *Gg*, *Gallus gallus*; *Cp*, *Cynops pyrrhogaster*; *Xl*, *Xenopus laevis*; *Pm*, *Petromyzon marinus*; *Lc*, *Latimeria chalumnae*; *Ga*, *Gasterosteus aculeatus*; *Om*, *Oncorhynchus mykiss*; *Dr*, *Danio rerio*; *Cag*, *Carassius auratus gibelio*

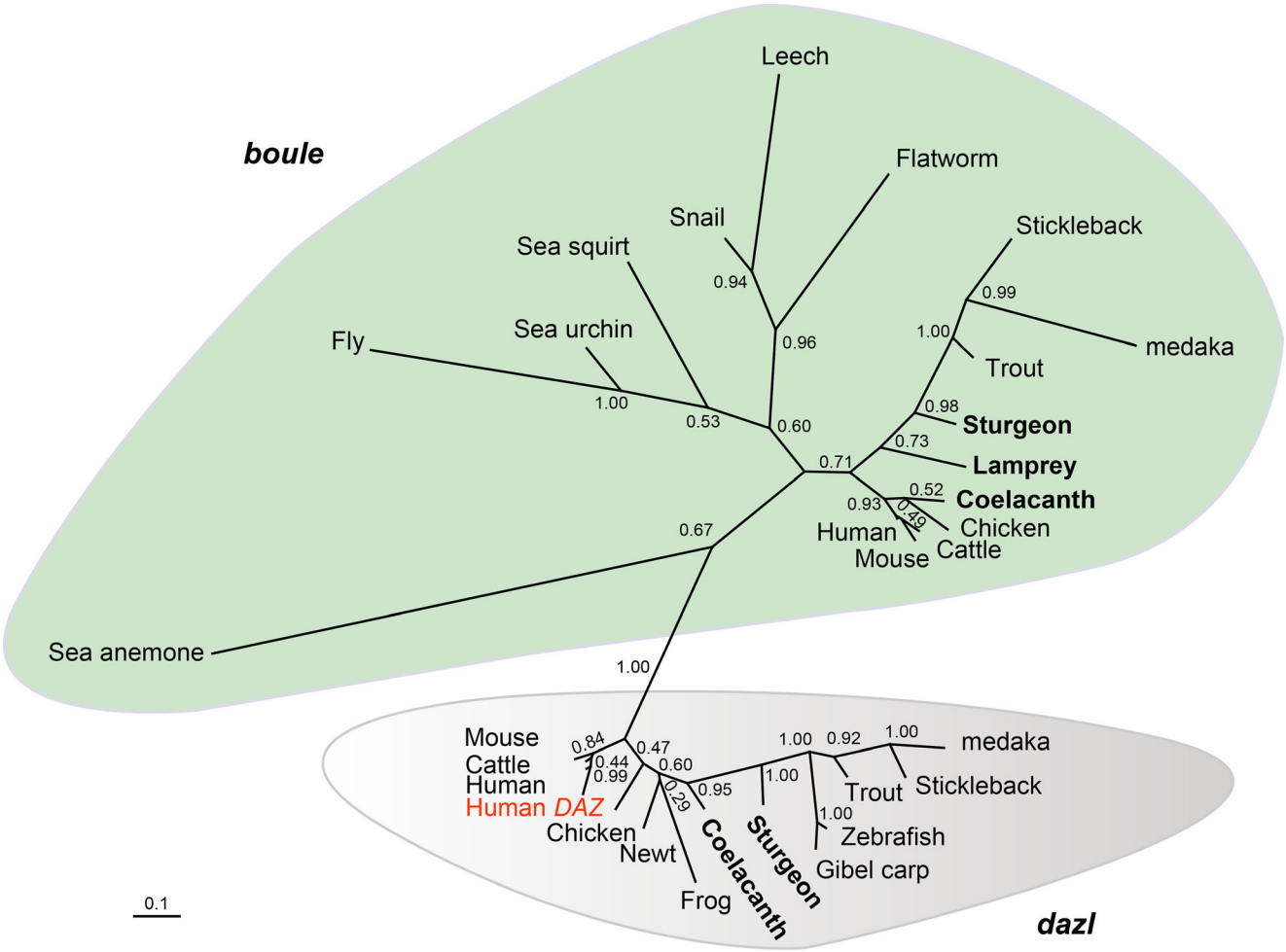

Fig. 3 Phylogenetic tree of *DAZ* family genes using Bayesian inference. Numbers near the nodes represent posterior probability values. The sequences were obtained from GenBank and Ensembl database. Human (BOULE: AAK58689, AF272858; DAZL: AAH27595, BC027595; DAZ: NP_004072, NM_004081); Cattle (BOULE:NP_001095585, NM_001102115; DAZL: NP_001075194, NM_001081725); Mouse (BOULE:NP_083543, NM_029267; DAZL: NP_034151, NM_010021); Chicken (BOULE: XP_421917, XM_421917; DAZL: AAO26019, AY211387); Newt (Dazl: BAD38676, AB164065); Frog (Dazl: NP_001081772, NM_001088303); Lamprey (Boule: ENSPMAP00000010046, ENSPMAT00000010090); Coelacanth (Boule: ENSLACP00000018009, ENSLACT00000018140; Dazl: ENSLACP00000003238, ENSLACT00000003268); Medaka (Boule: NP_001157989, NM_001164517; Dazl: AAX94053, AY973274); Stickleback (Boule: ENSGACP00000019047, ENSGACT00000019085; Dazl: ENSGACP00000009272, ENSGACT00000009292); Trout (Boule: ADW41783, HQ696915; Dazl: ADW41782, HQ696914); Zebrafish (Dazl: NP_571599, NM_131524); Gibel carp (Dazl: ACN94469, FJ774072); Sea squirt (Boule: XM_002124582); Sea urchin (Boule: XM_003727838); Fly (Boule: AAC47133, DMU51858); Nematode (Boule: BAA88577); Flatworm (Boule: XM_002575473); Snail (Boule: EV821571.2); Leech (Boule: EY313120); Sea anemone (Boule: XM_001635170)

barely detectable in sperm. On the other hand, Dazl exhibited an expression pattern overlapping with but different from, Boule. The signal was observed in spermatogonia and spermatocytes, barely detectable in spermatid and undetectable in sperm.

Co-localization of Dazl with Balbiani body/mitochondrial cloud

In order to validate the co-localization of Chinese sturgeon Dazl and MC, mitochondrial probe MitoTracker and Dazl antiserum were used to stain oocytes. As shown in Fig. 7, the two signals appeared not to completely overlap, suggesting that Dazl partially co-localized with the MC.

Discussion

The *DAZ* gene family is essential for germ line development in animals examined so far. In this study, we isolated *boule* and *dazl* genes in the Chinese sturgeon and analyzed their RNA and protein expressions in somatic tissues. Furthermore, their cellular and subcellular distribution in the gonad was investigated for the first time, to simultaneously reveal mRNA and protein expression patterns of *boule* and *dazl* in fish.

Based on the multiple amino acids sequence alignments of the RRM domain of Boule and Dazl, two RNP motifs, RNP1 and RNP2, are highly conserved in various species. Moreover, the molecular tree based on either the Boule or Dazl RRM

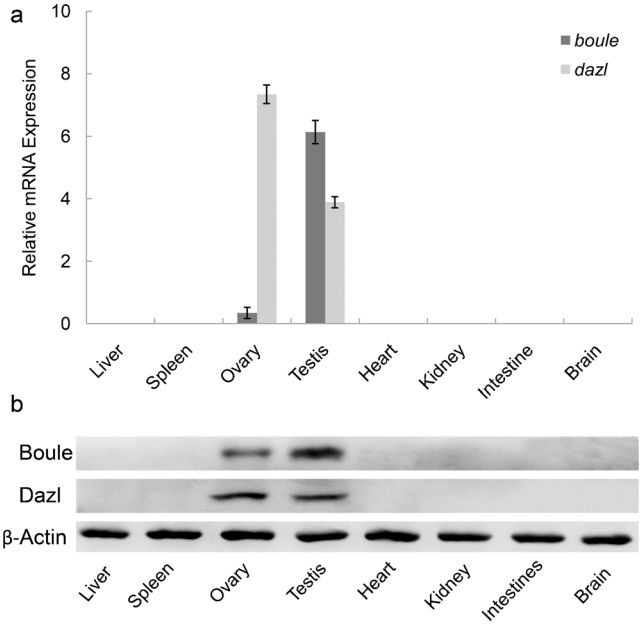

Fig. 4 Expression pattern of mRNA and protein of Chinese sturgeon *boule* and *dazl* in various tissues. **a** Tissue distribution patterns of *boule* and *dazl* evaluated by quantitative real-time PCR. The data were normalized to *β-actin* mRNA and represented mean ± SD of three separate experiments. The data are expressed as the mean fold change relative to the *β-actin* gene. **b** Western blot detection of Boule and Dazl proteins in immature individuals' tissues. Ubiquitous expression of β-Actin was used as the internal control

domain does not fully reflect the organism relationship. This may be due to the fact that Boule and Dazl are not appropriate for analysis of the phylogenetic relationship of species with close kinship. Several lines of evidence, including protein sequence and structure and phylogenetic sequence comparisons, strongly support the view that the *boule* and *dazl* genes of Chinese sturgeon and coelacanth are orthologous to the known *boule* and *dazl*, respectively. Additionally, from a strictly evolutionary viewpoint, the fact that *boule* and *dazl* genes are identified both in more derived ray-fins and tetrapods indicates that they are also present in sturgeon and coelacanth.

The *boule* gene is considered the ancestor of the *DAZ* gene family, giving rise to *dazl* through gene duplication during early vertebrate lineage (Xu et al. 2001). With the identification of *boule* and *dazl* in medaka (Xu et al. 2009) and trout (Li et al. 2011), together with the lack of a *dazl* homolog in jawless vertebrates and cartilaginous fish, Shah et al. (2010) believed that *dazl* most likely originated in the ancestral lineage of bony fish before the appearance of tetrapods. Furthermore, in the present study, *dazl* is found in Chinese sturgeon and coelacanth, belonging to Acipenseriformes and Coelacanthiformes, respectively, giving further evidence to demonstrate that *dazl* originates after the divergence of bony fish from cartilaginous fish but before the emergence of Actinistia. In addition, a *boule* homolog has been identified in major branches of fishes, such as *Astyanax mexicanus* (ENSAMXP00000005184), *Xiphophorus maculates* (ENSXMAP00000015769), *Lepisosteus oculatus* (ENSLOCP00000004776) and *Oreochromis niloticus* (ENSONIP00000019209). However, it has not been found in zebrafish (*Danio rerio*). Since *Danio rerio* and *Astyanax mexicanus* belong to Cypriniformes, it is likely that the lack of a zebrafish *boule* homolog may be ascribed to the incomplete genome.

A significant finding in this study is to give direct evidence to corroborate that sturgeon BB/MC disperses in the cytoplasm of early developing oocytes. Previous works have

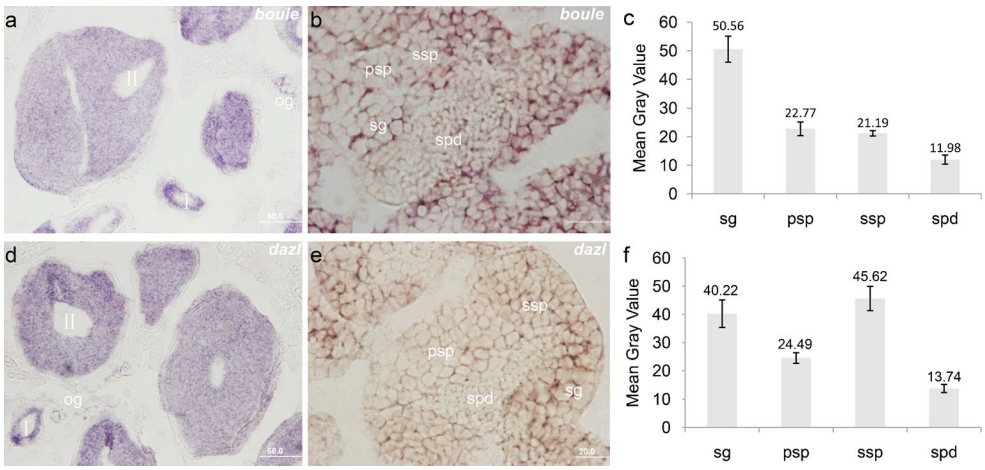

Fig. 5 Expression of Chinese sturgeon *boule* and *dazl* mRNA in the gonad. The ovarian and testis cryosections were hybridized to the *boule*-specific and *dazl*-specific antisense RNA probes, respectively and the signals were detected by alkaline phosphatase staining with BCIP/NBT as the chromogenic substrate. **a**, **d** Magnification view showing the ovary of 2.5-year-old Chinese sturgeon (*I* to *II*) hybridized by the *boule*-specific and *dazl*-specific antisense RNA probes, respectively. **b**, **e** Testis sections hybridized by the *boule*-specific and *dazl*-specific antisense RNA probes, respectively. (**c**, **f**) Statistical data of *boule* and *dazl* expression in the testis analyzed by Image J, respectively. Three cells are taken for every type of germ cell. The mean gray value is used. *og* oogonia, *sg* spermatogonia, *psp* primary spermatocytes, *ssp* secondary spermatocytes, spd, spermatid. *Bars* (**a**, **d**) 50 μm, (**b**, **e**) 20 μm

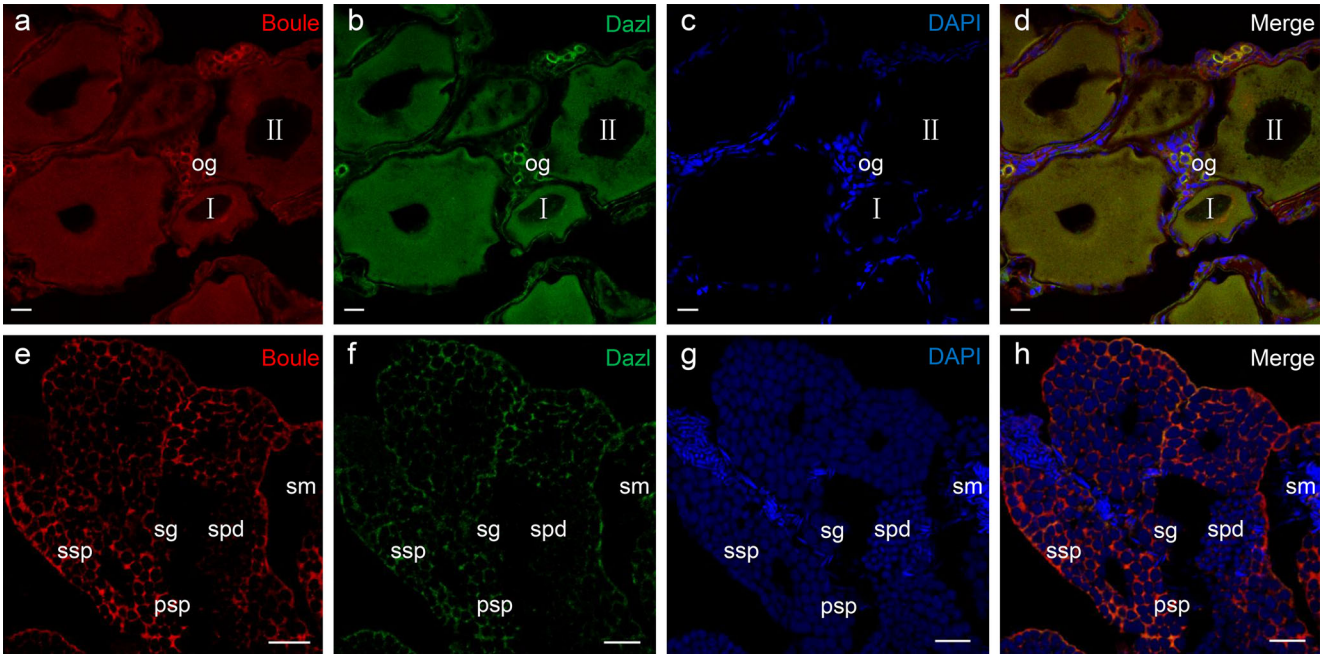

Fig. 6 Immunofluoresence co-localization of Chinese sturgeon Boule and Dazl in the gonad. Gonad sections were stained green for Dazl protein, red for Boule protein and blue for DAPI. **a**, **b** The ovary of 2.5 year-old Chinese sturgeon stained by the anti-Dazl and anti-Boule antibodies, respectively. **c** Nuclei were stained blue by DAPI. **d** Merge of Dazl protein signal, Boule protein signal and DAPI staining. **e**, **f** The adult testis of Chinese sturgeon stained by the anti-Dazl and anti-Boule serums, respectively. **g** Nuclei were stained blue by DAPI. **h** Merge Dazl protein signal, Boule protein signal and DAPI staining. *og* oogonia, *sg* spermatogonia, *psp*, primary spermatocytes, *ssp* secondary spermatocytes, *spd* spermatid *sm* sperm. *Bars* 20 μm

shown that *dazl* RNA of *Xenopus* (Chang et al. 2004), zebrafish (Kosaka et al. 2007), gible carp (Peng et al. 2009),

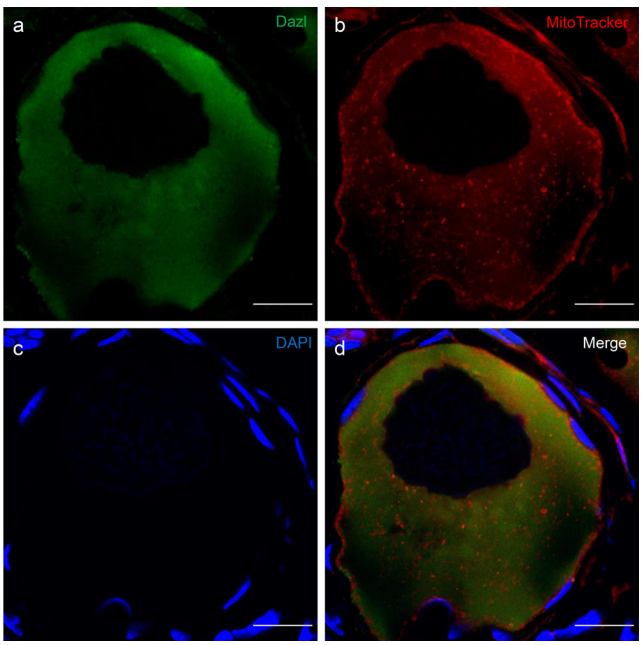

Fig. 7 Co-localization of Chinese sturgeon Dazl and Balbiani body/Mitochondrial cloud. **a** Dazl antiserum staining of the ovary of Chinese Sturgeon. **b** MitoTracker staining of Balbiani body/mitochondrial cloud of Chinese Sturgeon ovary. **c** Nuclei were stained blue by DAPI. **d** Merge Dazl protein signal, Balbiani body/Mitochondrial cloud and DAPI staining. *Bars* 20 μm

medaka (Xu et al. 2009) and trout (Li et al. 2011) are concentrated within the BB/MC, a spherical-shaped structure. Nonetheless, in *Acipenser gueldenstaedtii* (Zelazowska et al. 2007), oocytes contain two different zones of cytoplasm, the homogeneous ooplasm and granular ooplasm, with the granular ooplasm corresponding to the BB/MC. In sharp contrast to *Xenopus* and teleost BB/MC, by electron microscopy, in situ hybridization and immunostaining (Zelazowska et al. 2007), the BB/MC surrounds the germinal vesicle and homogeneous ooplasm distributes in the oocyte periphery at stage I. At stage II, the BB/MC extends toward the oocyte plasma. At stage III, the BB/MC and homogeneous ooplasm are combined and evenly distributed in the cytoplasm of oocytes. The Chinese sturgeon BB/MC, however, seems to diffuse in the cytoplasm of early developing oocytes by in situ hybridization and MitoTracker staining, similar to the Russian sturgeon BB/MC distribution at stage II and stage III, while the Russian sturgeon BB/MC is concentrated around the germinal vesicle at stage I. There was little difference in the characterization of BB/MC distribution in Russian and Chinese sturgeon, because the antisense probes taken by Zelazowska et al. (2007) belonged to *Drosophila* and *Xenopus*, not itself and were hydrolyzed to fragments of 20–50 bp, which would include much non-specific hybridization, resulting in abundant germ plasm RNA concentrated around the germinal vesicle. In summary, based on in situ hybridization of germ plasm RNA, *dazl* and colocalization MitoTracker and Dazl, we confirmed the

sturgeon BB/MC distribution in the cytosolic of early developing oocytes and partial co-localization with *dazl*.

The germline-specific expression of the *DAZ* family genes is usually conserved, especially the bisexual mitotic and meiotic germline expression of *dazl* homologs (Fig. 8). In ovary of fish, *dazl* RNA and protein persist throughout oogenesis (Li et al. 2011; Peng et al. 2009; Xu et al. 2009), with the most intense protein signal in oogonia (Peng et al. 2009; Xu et al. 2007). In this study, the expression of Chinese sturgeon *dazl* was investigated in early developing oocytes. The *dazl* signal was faint in oogonia and dramatically increased in primary oocytes, while its protein was abundant in the cytoplasm of oogonia and remained high in primary oocytes, similar to that of medaka (Fig. 7). On the other hand, in vertebrate testis, *dazl* homologs are generally transcribed through spermatogonia and spermatocytes and into early round spermatids (Fig. 8). Such a protein expression pattern is found in frog (Mita and Yamashita 2000), mouse (Ruggiu et al. 1997) and human (Reijo et al. 2000; Xu et al. 2001). In Chinese sturgeon, *dazl* expression is more similar to that of medaka, as opposed to trout, in which *dazl* persists in postmeiotic spermatids (Fig. 8). The *dazl* signal was observed in spermatogonia and primary spermatocytes, increased slightly in secondary spermatocytes and was detectable in spermatid, with similar expression of Dazl protein. Moreover, its subcellular localization is in cytoplasm, not like that of mouse and human, which is nuclear in gonocytes and translocates from the nuclei of spermatogonia into the cytoplasm of spermatocytes (Reijo et al. 2000). In summary, in spite of the absence of sufficient RNA and protein information at each stage and slight variations in species-specific expression, a common pattern of *dazl* RNA and protein expression in the mitotic and meiotic phases of both female and male gametogenesis is conserved (Fig. 8), which may represent the ancient characteristics of the vertebrate *dazl* gene.

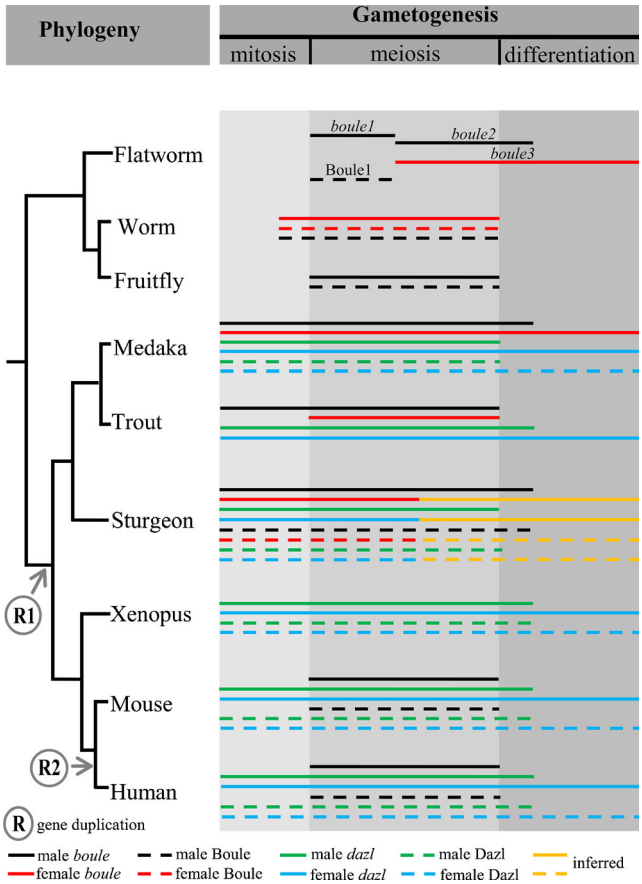

Fig. 8 Comparison of the *DAZ* family genes expression during gametogenesis. *Left* phylogeny. The ancient member, *boule*, is present in all metazoans, while *dazl* occurs in vertebrates, and *DAZ* is restricted to catarrhine primates. The duplications of *DAZ* family genes are indicated by R1 and R2. The depicted phylogenetic tree only exhibits the relationship between genera but not exact evolutionary distances. *Right* gametogenic expression. The expression pattern of sex specificity is represented by the length of *horizontal lines* with different colors (male *boule*, *solid black line*; female *boule*, *solid red line*; male *dazl*, *solid green line*; female *dazl*, *solid blue line*; inferred, *orange line*; proteins, *dashed lines*). Major stages of germ cell development are depicted in a timeline for the *boule* and *dazl* gene expression. Diagram was created by the authors or redrawn based on the references (Eberhart et al. 1996; Houston et al. 1998; Karashima et al. 2000; Kuales et al. 2011; Li et al. 2011; Maruyama et al. 2005; Mita and Yamashita 2000; Reijo et al. 2000; Ruggiu et al. 1997; Shah et al. 2010; Vangompel and Xu 2011; Xu et al. 2001, 2007, 2009)

However, the expression of the third family member, *boule*, is more divergent than that of the other two (Fig. 8). It exhibits predominantly transcription in the testes of fly (Eberhart et al. 1996), sea urchins (Shah et al. 2010), chicken (Shah et al. 2010) and mice (Shah et al. 2010), ovarian expression in nematode (Karashima et al. 2000) and both testis and ovary expression in flatworm (Kuales et al. 2011), medaka (Xu et al. 2009) and trout (Li et al. 2011). By the analysis of the quantitative real-time PCR and western blot, Chinese sturgeon *boule* RNA and proteins were detected in both testis and ovary, similar to other fishes, suggesting the conservation of the bisexual expression of *boule* in fish (Fig. 8). In testis, *boule* shows meiotic-specific expression in fly (Eberhart et al. 1996), mouse (Xu et al. 2001) and human (Xu et al. 2001) and meiotic-preferential expression in medaka (Xu et al. 2009) and trout (Li et al. 2011). The present study found mitotic and meiotic expression of *boule* RNA in Chinese sturgeon, similar to medaka and trout. The signal of Chinese sturgeon *boule* peaked in spermatogonia, declined in primary and secondary spermatocytes and was faint in spermatid. Furthermore, Chinese sturgeon Boule proteins were found in spermatogonia and spermatocytes, reduced in spermatid and hardly identified in sperm, indicating that the *boule* mitotic and meiotic expression pattern in male is conserved in fish. In ovary, the nematode *boule* homolog, *daz-1*, is necessary for oogenesis (Karashima et al. 2000; Maruyama et al. 2005). In *Macrostomum lignano*, a third flatworm *boule* homolog, *Macbol3*, is present in later oocytes and RNAi treatment leads to aberrant egg maturation and possible sterility (Kuales et al.

2011). Subsequently, in vertebrates, a low level of mouse *Boule* RNA is found in the early embryonic gonads of both sexes and in the adult ovary but its protein is not detected (Shah et al. 2010; Vangompel and Xu 2011). In fish, medaka *boule* is expressed in both mitotic and meiotic stages in the ovary (Xu et al. 2009), while its counterpart in trout is only detected at the meiotic stage (Li et al. 2011). In the present study, Chinese sturgeon *boule* displayed expression similar to that in medaka (Fig. 8). In the early oocytes, both *boule* RNA and proteins were detected in oogonia and primary oocytes, which is the first report of vertebrate Boule protein in ovary. These results suggest that *boule* possibly functions in the ovary of a diverse range of animals.

Previously, Shah et al. (2010) proposed that urbilaterian *boule* had an ancestral function in male gametogenesis, which was lost during the evolution of the nematode lineage. However, they did not rule out the possibility of an additional ancestral ovarian function. Furthermore, as RNAi of *Macbol1* and *Macbol3*, respectively, block spermatocyte differentiation and result in female sterility in flatworm (Kuales et al. 2011), a primitive Protostomia, together with the first identification of Boule protein in the ovary of vertebrate, Chinese sturgeon, we suggest that the urbilaterian *boule* may also have had an ancestral function in oogenesis but lost the ovarian function in fly and testis function in nematode. Further function analysis of *boule* in other animal lineage, such as medaka and sea anemone, would help to elucidate its ancestral roles.

In conclusion, the present study identified *boule* and *dazl* genes in the Chinese sturgeon. This, together with the observation of the *boule* and *dazl* genes in the coelacanth, suggests that *dazl* originated after the divergence of bony fish from cartilaginous fish but before the emergence of Actinistia. Subsequently, by quantitative real-time PCR and western blot analysis, the RNA and protein expressions of *boule* and *dazl* are restricted to the gonad of both sexes. Furthermore, the cellular and subcellular localizations of *boule* and *dazl* in the gonad represent significant findings. First, the bisexual mitotic and meiotic germ cell-specific expression of *dazl* RNA and protein is conserved in vertebrates, while *boule* RNA and protein show mitotic and meiotic male expression and mainly display mitotic and meiotic expression in female, providing two germ cells markers to identify different stages of gametogenesis in Chinese sturgeon for germline engineering. Second, sturgeon Balbiani body/mitochondrial cloud is dispersed in the cytoplasm of early developing oocytes and partially co-localizes with Dazl. Finally, it is reasonable to infer that the urbilaterian *Boule* may also have an ancestral function in oogenesis.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31472286 and 31172413), the National High Technology Research and Development Program of China (2011AA100401) and the National Nonprofit Institute Research Grant of Freshwater Fisheries Research Center, Chinese Academy of Fisheries (2013JBFZ01).

References

Agulnik AI, Zharkikh A, Boettger-Tong H, Bourgeron T, McElreavey K, Bishop CE (1998) Evolution of the *DAZ* gene family suggests that Y-linked *DAZ* plays little, or a limited, role in spermatogenesis but underlines a recent African origin for human populations. Hum Mol Genet 7:1371–1377

Amemiya CT, Alföldi J, Lee AP, Fan S, Philippe H, MacCallum I, Braasch I, Manousaki T, Schneider I, Rohner N (2013) The African coelacanth genome provides insights into tetrapod evolution. Nature 496:311–316

Birstein VJ, Bemis WE, Waldman JR (1997) The threatened status of acipenseriform species: a summary. Environ Biol Fish 48:427–435

Chang P, Torres J, Lewis RA, Mowry KL, Houliston E, King ML (2004) Localization of RNAs to the mitochondrial cloud in *Xenopus* oocytes through entrapment and association with endoplasmic reticulum. Mol Biol Cell 15:4669–4681

Chen X, Wei Q, Yang D, Zhu Y (2006) Observations on the formation and development of the primary germinal tissue of cultured Chinese sturgeon, *Acipenser sinensis*. J Appl Ichthyol 22:358

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

Eberhart CG, Maines JZ, Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*. Nature 381:783–785

Gromoll J, Weinbauer GF, Skaletsky H, Schlatt S, Rocchietti-March M, Page DC, Nieschlag E (1999) The Old World monkey *DAZ* (Deleted in AZoospermia) gene yields insights into the evolution of the *DAZ* gene cluster on the human Y chromosome. Hum Mol Genet 8:2017–2024

Houston DW, King ML (2000) A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. Development 127:447–456

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 homologue of *Drosophila boule*. Development 125:171–180

Huang WJ, Lin YW, Hsiao KN, Eilber KS, Salido EC, Yen PH (2008) Restricted expression of the human DAZ protein in premeiotic germ cells. Hum Reprod 23:1280–1289

Johnson AD, Bachvarova RF, Drum M, Masi T (2001) Expression of axolotl *DAZL* RNA, a marker of germ plasm: widespread maternal RNA and onset of expression in germ cells approaching the gonad. Dev Biol 234:402–415

Karashima T, Sugimoto A, Yamamoto M (2000) *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. Development 127:1069–1079

King ML, Messitt TJ, Mowry KL (2005) Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biol Cell 97:19–33

Kosaka K, Kawakami K, Sakamoto H, Inoue K (2007) Spatiotemporal localization of germ plasm RNAs during zebrafish oogenesis. Mech Dev 124:279–289

Kuales G, De Mulder K, Glashauser J, Salvenmoser W, Takashima S, Hartenstein V, Berezikov E, Salzburger W, Ladurner P (2011) *Boule*-like genes regulate male and female gametogenesis in the flatworm *Macrostomum lignano*. Dev Biol 357:117–132

Lacerda SM, Costa GM, Campos-Junior PH, Segatelli TM, Yazawa R, Takeuchi Y, Morita T, Yoshizaki G, Franca LR (2013) Germ cell transplantation as a potential biotechnological approach to fish reproduction. Fish Physiol Biochem 39:3–11

Li M, Shen Q, Xu H, Wong FM, Cui J, Li Z, Hong N, Wang L, Zhao H, Ma B, Hong Y (2011) Differential conservation and divergence of

fertility genes *boule* and *dazl* in the rainbow trout. PLoS ONE 6: e15910

Maegawa S, Yasuda K, Inoue K (1999) Maternal mRNA localization of zebrafish *DAZ-like* gene. Mech Dev 81:223–226

Maruyama R, Endo S, Sugimoto A, Yamamoto M (2005) *Caenorhabditis elegans* DAZ-1 is expressed in proliferating germ cells and directs proper nuclear organization and cytoplasmic core formation during oogenesis. Dev Biol 277:142–154

Mita K, Yamashita M (2000) Expression of *Xenopus* Daz-like protein during gametogenesis and embryogenesis. Mech Dev 94:251–255

Peng JX, Xie JL, Zhou L, Hong YH, Gui JF (2009) Evolutionary conservation of *Dazl* genomic organization and its continuous and dynamic distribution throughout germline development in gynogenetic gibel carp. J Exp Zool B 312:855–871

Reijo RA, Dorfman DM, Slee R, Renshaw AA, Loughlin KR, Cooke H, Page DC (2000) DAZ family proteins exist throughout male germ cell development and transit from nucleus to cytoplasm at meiosis in humans and mice. Biol Reprod 63:1490–1496

Rengaraj D, Zheng YH, Kang KS, Park KJ, Lee BR, Lee SI, Choi JW, Han JY (2010) Conserved expression pattern of chicken *DAZL* in primordial germ cells and germ-line cells. Theriogenology 74:765–776

Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ (1997) The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389:73–77

Saxena R, Brown LG, Hawkins T, Alagappan RK, Skaletsky H, Reeve MP, Reijo R, Rozen S, Dinulos MB, Disteche CM, Page DC (1996) The D*AZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. Nat Genet 14:292–299

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108

Seboun E, Barbaux S, Bourgeron T, Nishi S, Agulnik A, Egashira M, Nikkawa N, Bishop C, Fellous M, McElreavey K, Kasahara M (1997) Gene sequence, localization, and evolutionary conservation of *DAZLA*, a candidate male sterility gene. Genomics 41:227–235

Shah C, Vangompel MJ, Naeem V, Chen Y, Lee T, Angeloni N, Wang Y, Xu EY (2010) Widespread presence of human *BOULE* homologs among animals and conservation of their ancient reproductive function. PLoS Genet 6:e1001022

Shan Z, Hirschmann P, Seebacher T, Edelmann A, Jauch A, Morell J, Urbitsch P, Vogt PH (1996) A *SPGY* copy homologous to the mouse gene *Dazla* and the *Drosophila* gene *boule* is autosomal and expressed only in the human male gonad. Hum Mol Genet 5: 2005–2011

Slee R, Grimes B, Speed RM, Taggart M, Maguire SM, Ross A, McGill NI, Saunders PT, Cooke HJ (1999) A human *DAZ* transgene confers partial rescue of the mouse *Dazl* null phenotype. Proc Natl Acad Sci U S A 96:8040–8045

Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 3:59–69

Vangompel MJ, Xu EY (2011) The roles of the *DAZ* family in spermatogenesis: more than just translation? Spermatogenesis 1:36–46

Vogel T, Speed RM, Ross A, Cooke HJ (2002) Partial rescue of the *Dazl* knockout mouse by the human *DAZL* gene. Mol Hum Reprod 8: 797–804

Wei Q, Fe K, Zhang J, Zhuang P, Luo J, Zhou R, Yang W (1997) Biology, fisheries, and conservation of sturgeons and paddlefish in China. Environ Biol Fish 48:241–255

Wei QW, Li LX, Du H, Zhang XY, Xiong W, Zhang H, Shen L, Wu JM, Zhang SH, Wang CY, Li CJ, Chai Y, Li XW, Qiao XM, Liu ZG, Gao YP, Gan F (2013) Research on technology for controlled propagation of cultured Chinese sturgeon (*Acipenser sinensis*). J Fish Sci China 20:1–11 (In Chinese)

Xia W, Zhou L, Yao B, Li CJ, Gui JF (2007) Differential and spermatogenic cell-specific expression of *DMRT1* during sex reversal in protogynous hermaphroditic groupers. Mol Cell Endocrinol 263: 156–172

Xu EY, Moore FL, Pera RA (2001) A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. Proc Natl Acad Sci U S A 98:7414–7419

Xu EY, Lee DF, Klebes A, Turek PJ, Kornberg TB, Reijo Pera RA (2003) Human BOULE gene rescues meiotic defects in infertile flies. Hum Mol Genet 12:169–175

Xu H, Li M, Gui J, Hong Y (2007) Cloning and expression of medaka *dazl* during embryogenesis and gametogenesis. Gene Expr Patterns 7:332–338

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

Ye H, Chen X, Wei Q, Zhou L, Liu T, Gui J, Li C, Cao H (2012) Molecular and expression characterization of a *nanos1* homologue in Chinese sturgeon, *Acipenser sinensis*. Gene 511:285–292

Zelazowska M, Kilarski W, Bilinski SM, Podder DD, Kloc M (2007) Balbiani cytoplasm in oocytes of a primitive fish, the sturgeon *Acipenser gueldenstaedtii*, and its potential homology to the Balbiani body (mitochondrial cloud) of *Xenopus laevis* oocytes. Cell Tissue Res 329:137–145