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Differential expression of the *Brunol/CELF* family genes during *Xenopus laevis* early development

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ABSTRACT The BRUNOL/CELF family of RNA-binding proteins plays important roles in post-transcriptional regulation and has been implicated in several developmental processes. In this study, we describe the cloning and expression patterns of five *Brunol* genes in *Xenopus laevis*. Among them, only *Brunol2* is maternally expressed and the zygotic expression of the other four *Brunol* genes starts at different developmental stages. During *Xenopus* development, *Brunol1, 4-5* are exclusively expressed in the nervous system including domains in the brain, spinal cord, optic and otic vesicles. *Brunol2* and *3* are expressed in both the somatic mesoderm and the nervous system. *Brunol2* is also extensively expressed in the lens. In transfected Hela cells, BRUNOL1, 2 and 3 proteins are localized in both the cytoplasm and the nucleus, while BRUNOL4 and 5 are only present in the cytoplasm, indicating their different functions.

KEY WORDS: *Brunol, CELF, Xenopus, expression pattern*

The BRUNOL (Bruno-like) family of RNA-binding proteins has been implicated in several post-transcriptional regulatory processes including pre-mRNA alternative splicing, mRNA stability and translation. The family was named due to their protein sequence similarity to *Drosophila melanogaster* Bruno (Good *et al.*, 2000). The same family was also named CELF (CUG-BP1 and ETR-3 Like Factors) (Ladd *et al.*, 2001). For the correspondence between these two systems please refer to Barreau *et al*. (2006) and Brimacombe and Ladd (2007). BRUNOL family proteins contain three RNA recognition motifs (RRMs), two in the N-terminal region and one in the C-terminal region. The RRMs show a high degree of sequence similarity but the sequences of the linker region between the second and third RRMs are less conserved (Barreau *et al.*, 2006).

Mammalian BRUNOL family of proteins contains six members. A phylogenetic analysis suggests that the human BRUNOL proteins fall into two distinct subfamilies: one contains BRUNOL2, 3 (CUG-BP1 and ETR-3), and the other BRUNOL1, 4-6 (CELF3-6) (Ladd and Cooper, 2004). *Brunol2* and *3* are broadly expressed in adult human and mice tissues, with strong expression in the heart, skeletal muscle, and brain (Good *et al.*, 2000; Ladd *et al.*, 2001; Ladd *et al.*, 2004). In contrast, *Brunol1, 4*, *5* and 6 are mainly expressed in the brain (Good *et al.*, 2000; Ladd *et al.*, 2001; Ladd *et al.*, 2004; Yang *et al.*, 2007) with *Brunol6* also in the kidney and testis (Ladd and Cooper, 2004). In chicken, 5 *Brunol* genes have been isolated, with *Brunol1* (*CELF3*) missing. Among them, *Brunol2* and *3* (*CUG-BP1* and *ETR-3*) are broadly expressed in the early embryos, while *Brunol4-6* (*CELF4-6*) are restricted primarily to the nervous system (Brimacombe and Ladd, 2007). In addition to *Bruno*, there are two more *Brunol* genes in *Drosophila*, *Bru-2* and *3*, of which the expressions and functions are still unknown (Delaunay *et al.*, 2004). There are two *Brunol* homologous genes in *C. elegans*, *etr-1* (homologous to *Brunol2*, *3*) and *unc-75* (homologous to *Brunol1*, *4-6*). *etr-1* is expressed in muscle cells (Milne and Hodgkin, 1999) while *unc-75* in the nervous system (Loria *et al.*, 2003). These data suggest that the two subfamilies of *Brunol* genes arose very early in evolution and their expression patterns and possible functions are somehow conserved during evolution. Functional studies in *C. elegans*,

Abbreviations used in this paper: PBS, phosphate buffered saline; PCR, polymerase chain reaction; RRM, RNA recognition motif; WMISH, whole mount *in situ* hybridization.

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Drosophila and mouse have revealed the involvement of BRUNOL proteins in the neural, muscle and germ cell development (Loria *et al*., 2003; Milne and Hodgkin, 1999; Timchenko *et al*., 2004; Kim-Ha *et al*., 1995; Kress *et al*., 2007; Dev *et al*., 2007).

In *Xenopus*, two members of *Brunol* genes have been reported previously. *Brunol1*, also named *etr-1*, was found in a screen of genes expressed in noggin-induced neural tissue (Knecht *et al.*, 1995). *Xenopus* BRUNOL2 (also known as EDEN-BP) was firstly isolated as a maternal factor mediating sequence-specific mRNA deadenylation in early *Xenopus* embryos (Paillard *et al.*, 1998). Recently, more than 150 maternal mRNAs from *Xenopus tropicalis* egg extracts have been identified as potential BRUNOL2 targets (Graindorge *et al.*, 2008), suggesting an important role of BRUNOL2 in early embryonic development. *Xenopus Brunol2* is also implicated in the segmentation process. Inactivation of *Brunol2* causes severe segmentation defects, and the expression of segmentation markers in the Notch signalling pathway is disrupted (Gautier-Courteille *et al.*, 2004).

In this study, we have cloned three new *Brunol* genes (*Brunol3-5*) as well as different paralogs of *Brunol1* and *2* of *Xenopus laevis* and systematically studied their expression patterns during early embryogenesis. The sub-cellular localization of the different BRUNOL proteins was also compared. The results suggest overlapping but different roles of the *Brunol* genes in *Xenopus* development.

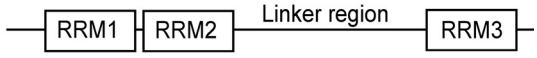
Results and Discussion

Isolation and phylogenetic analysis of Xenopus Brunol *genes*

The *Xenopus laevis Brunol1a* (*etr-1*) and *Brunol2a* (*EDEN-BP*) have been reported previously (Knecht *et al.*, 1995; Paillard *et al.*, 1998). Since *X. laevis* is pseudotetraploid, there often exit two closely related paralogs for each gene (Hellsten *et al*., 2007). In our attempt to clone the *X. laevis Brunol* genes, we have found different paralogs for both *Brunol1* and *2*, designated *Brunol1b* and *2b*, showing 98% and 92% identity with *Brunol1a* and *2a* respectively. Two *Brunol3* paralogs, designated *Brunol3a* and *3b*, were isolated, with 88% sequence identity. Compared with BRUNOL3a, BRUNOL3b missed about 44 amino acids in the linker region, likely due to alternative splicing. *X. laevis Brunol4* and *5* were cloned by PCR. One additional isoform of *Brunol5* (*Brunol5a*) was also isolated which missed one 49 base-pair exon to produce a premature stop codon, resulting in a truncated protein of only 132 amino acids. We failed to clone the *Xenopus Brunol6* using primers according to conserved sequence among vertebrate *Brunol6* mRNAs. Using mouse *Brunol* mRNA sequence queries, we could find only 5 chromosome loci in *X. tropicalis* genome. No ESTs of potential *Brunol6* could be found either in *X. laevis* or *tropicalis*. The predicted partial sequence listed as *X. tropicalis* BRUNOL6 (CELF6) in Brimacombe and Ladd (2007) was proved to be the C-terminal part of BRUNOL1. It is possible that *Brunol6* is missing in *Xenopus*. In chick, *Brunol1* (CELF3) is missing due to a deletion event. The *Xenopus* BRUNOL proteins all contained three conserved RRMs with a linker region between the second and third one (Fig. 1A). Among the 5 *Xenopus* BRUNOL proteins, BRUNOL2 and BRUNOL3 proteins are more closely related with an overall sequence identity of 72%. BRUNOL1, 4 and 5 form another sub-group and the average sequence identity among them was about 66%. The overall sequence identity between the two sub-groups was below 45% (Fig. 1B).

Using MEGA program (Kumar *et al.*, 2004), we studied the phylogenetic relationship of the BRUNOL proteins in human,

A



B

BRUNOL	1	2	3	4	5
1	100	41	44	71	64
2		100	72	42	43
3			100	44	45
4				100	67
5					100

C

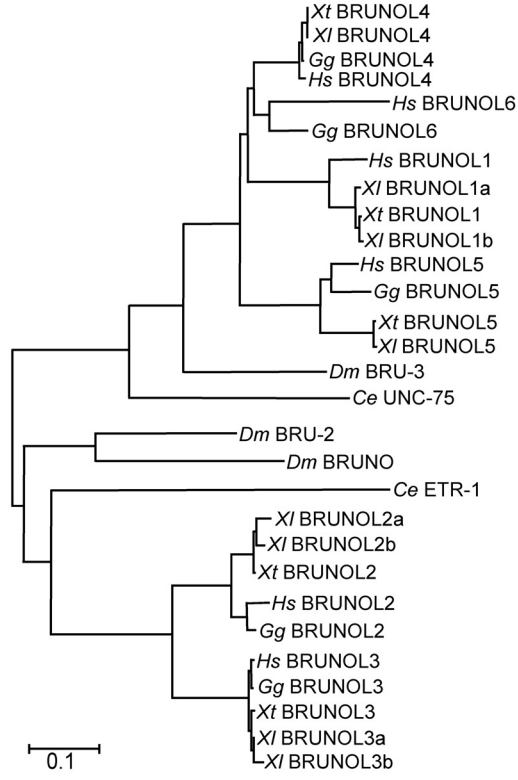


Fig. 1. Phylogenetic analysis of *Xenopus* BRUNOL proteins. (A) *The representative structure of* Xenopus *BRUNOL proteins.* **(B)** *Sequence identities of the* X. laevis *BRUNOL proteins. For BRUNOL proteins that have paralogs, the average values of the paralogs were shown.* **(C)** *Phylogenetic analysis of BRUNOL family proteins. ClustalW alignment, Poisson correction model, and Bootstrap test were used for the neighbor-joining (NJ) tree construction. The sequences of* X. laevis *BRUNOL3b, 4, 5, 5a have been deposited in GenBank under accession numbers EU743743-EU743746. Other accession nos. were as follows:* Xenopus laevis *(*Xl*) BBRUNOL1a* AAA81375, *BRUNOL1b* AAH46942, *BRUNOL2a* AAH70706, *BRUNOL2b* NP_001084196, *BRUNOL3a* AAB09041*;* Xenopus tropicalis *(*Xt*) BBRUNOL1* BC154063, *BRUNOL2* NP_001017152, *BRUNOL3* NP_001096417, *BRUNOL4* AAI21491, *BRUNOL5* AAI25740*;* Drosophila melanogaster (Dm) *Bruno* NP_723738, *BRU-2* NP_788039, *BRU-3* NP_729923. *The BRUNOL protein sequences of human (*Homo sapiens, Hs*), chicken* (Gallus gallus, Gg) *and* Caonorhabditis elegans (Ce) *were used as in Brimacome and Ladd (2007).*

chick, *X. laevis* and *tropicalis, Drosophila* and *C. elegans* (Fig. 1C). The BRUNOL proteins fell into two sub-groups based on their overall sequence similarity, one contained BRUNOL2 and 3, the other contained BRUNOL1 and 4-6. The *Drosophila* and *C. elegans* BRUNO proteins also fitted into the two BRUNOL subfamilies, suggesting an early diversification of the two subfamilies during evolution.

Temporal expression of Xenopus Brunol *genes*

To investigate the temporal expression patterns of the *Brunol* genes in *X. laevis*, a series of RT-PCR analysis was carried out (Fig. 2A). *Brunol2a* and *b* were detected strongly maternally as reported previously (Gautier-Courteille *et al.*, 2004). Their expression was weak at the beginning of the neurula stage and became stronger at later stages. All the other *Brunol* genes were not maternally expressed and their expression started at different developmental stages. *Brunol1* began to express at about stage 11 and increased with development. From stage 19 on, it kept strongly expressed. The transcripts of *Brunol3a* and *b* could be detected weakly at stage 19 and kept expressed at later stages. *Brunol4* and *5* started to express at stage 30 and 25 respectively and were both strongly expressed at later stages.

Different paralogs of the same *Brunol* genes showed the same temporal expression patterns during development, although their expression levels were slightly different. Since the sequences of *Brunol1a* and *b* were 98% identical, it was difficult to design PCR primers to distinguish them. They differed in one fragment in which *Brunol1b* contained two additional NcoI restriction sites. This fragment was amplified by PCR and after NcoI digestion, it was able to distinguish the two paralogs. At stages 11 to 30, the expression level of *Brunol1a* was stronger than *Brunol1b* with a ratio of about 2.5:1. At stages 36 and 43, the two paralogs were expressed at similar levels (Fig. 2B).

Spatial expression of Brunol *genes during* Xenopus laveis *early embryogenesis*

To avoid cross reaction, the sequences corresponding to the less conserved linker regions between RRM2 and 3 were chosen for probes preparation. For the different paralogs of the same gene, it was unable to distinguish the expression patterns of individual paralogs using these probes due to their high sequence similarities.

The temporal expression patterns of the *Brunol genes* from the WMISH results fitted well with the RT-PCR analysis. *Brunol2* was the only one maternally expressed and no expression was detected for *Brunol1*, *3*, *4* and *5* till the end of gastrulation.

As reported previously, *Brunol1* was specifically expressed in the neural tissue and stronger in the anterior region (Fig. 3A, Knecht *et al.*, 1995). At stage 13 and 14, weak expression of *Brunol1* was detected in the anterior neural plate region as well as two patches lateral to the brain region, representing the presumptive trigeminal placodes (Fig. 3Aa-d). From stage 16 to 25, *Brunol1* was more abundantly expressed in the whole neural tube but weak in the roof plate and floor plate regions (Fig. 3Ae-k). At stage 30, *Brunol1* was strongly expressed in the forebrain, midbrain, hindbrain, the epiphysis, spinal cord and optic vesicles, but not at mid-hindbrain boundary (Fig. 3Al). At stage 36, its expression remained strong in the above regions and appeared also in the otic vesicles (Fig. 3Am-p). In the eyes, it was strongly expressed in the retinas (Fig. 3 Ao).

Maternal *Brunol2* mRNA has been reported to distribute homogeneously in the early *Xenopus* embryos (Gautier-Courteille *et al.*, 2004) and vegetally in zebrafish (Suzuki *et al.*, 2000). In our study, however, *Brunol2* transcripts were detected at the animal poles in the unfertilized eggs and cleavage and blastula stage embryos (Fig. 3Ba and data not shown). During gastrulation, it was expressed in the ectoderm but not in the yolk plug region (Fig. 3Bb). At stage 13, it was broadly expressed in the whole embryo (Fig. 3Bc). At stages 14 and 16, its expression became confined in the para-axial mesoderm and the neural plate (Fig. 3Bd-g). At stages 19 and 26, it was expressed in the somites and neural tube (Fig. 3Bh-m). At stage 19, it was also detected in the trigeminal placodes and the presumptive eye fields (Fig. 3Bi). At tailbud stages, *Brunol2* was expressed in the brain, spinal cord, optic and otic vesicles and the branchial arches (Fig. 3Bn-s). It was extensively expressed in the lens (Fig. 3Bq), which was also the case for zebrafish *Brunol2* (Suzuki *et al.*, 2000), suggesting a conserved role for BRUNOL2 in lens development. *Brunol2* remained weakly expressed in the somites in the trunk region and strongly in the tail region (Fig. 3Bo,p,s).

Brunol3 began to be expressed at about stage 15 in the paraaxial mesoderm (data not shown). Its expression became clear in the somitic mesoderm (Fig. 3Ca,b) at stage 18 and remained strong in the somites through stages 25 to 30 (Fig. 3Cc,d,e). At stage 25, *Brunol3* began to express also weakly in the head region (Fig. 3Cc). In the nervous system, *Brunol3* was detected in patches in the midbrain, hindbrain, anterior spinal cord and optic vesicles at stage 30, but was absent at the midbrain-hindbrain boundary (Fig. 3Ce). At stage 36, the expression of *Brunol3* in the somites became very weak and clear only in the tail region (Fig. 3Cf,g,j). In the nervous system, its expression remained strong in patches in the brain region and also in the optic and otic vesicles

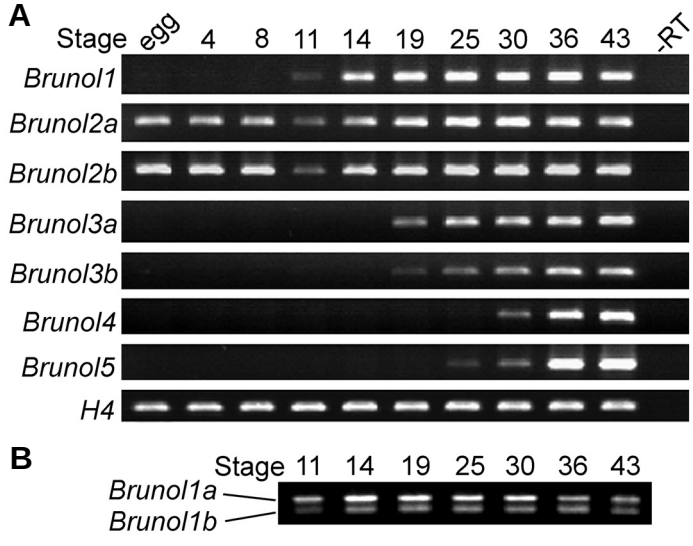


Fig. 2. Temporal expression of *Xenopus Brunol* genes. (A) *RT-PCR analysis of the developmental expression of the* Xenopus Brunol *genes. egg: unfertilized egg. Histone 4 (H4) was used as a loading control.* **(B)** *The* Brunol1 *PCR products after NcoI digestion, showing the differential expression levels of* Brunol1a *and* 1b.

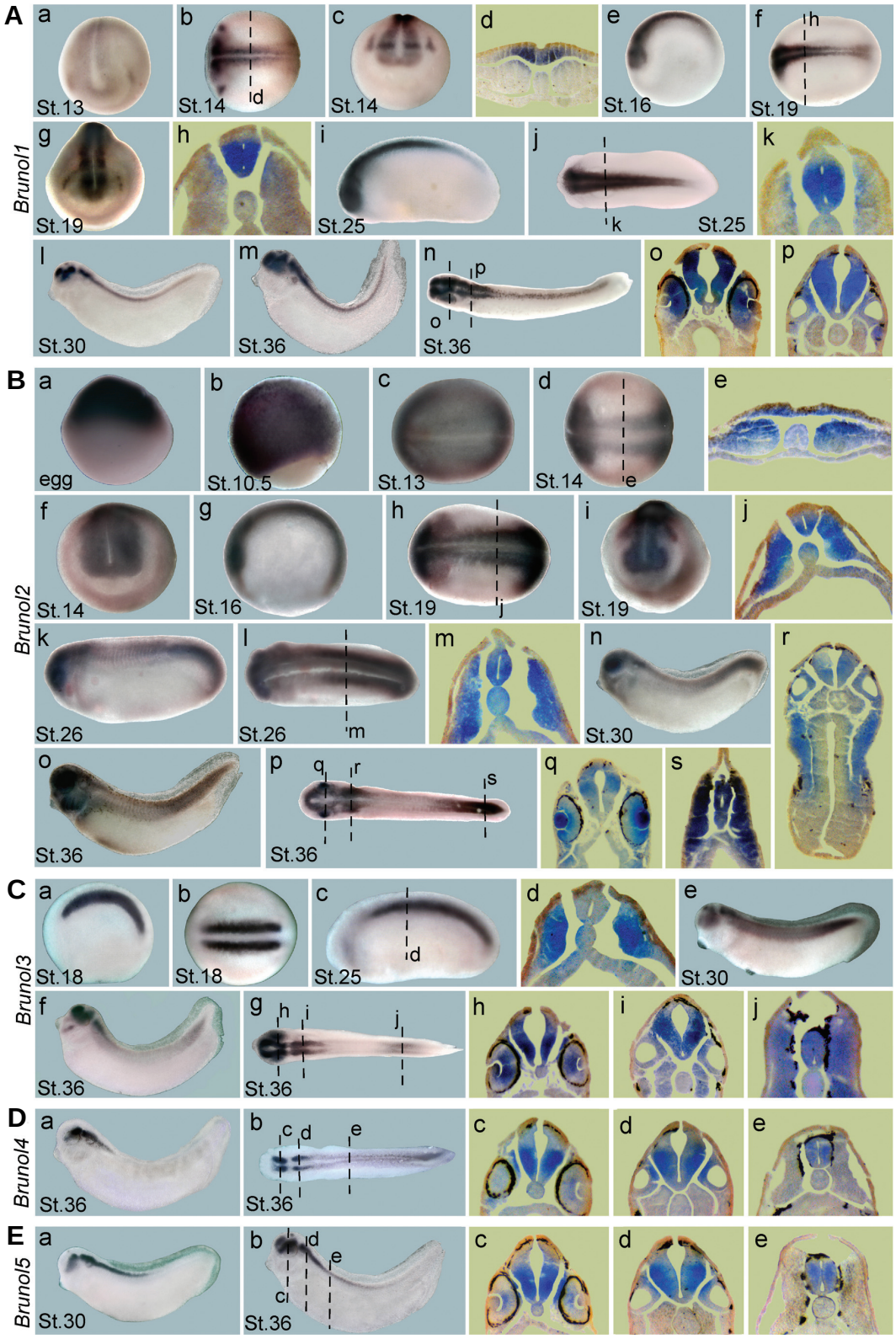


Fig. 3. Embryonic expression of the *X. laevis Brunol* genes at the indicated stages. (A) Brunol1, *(a,c,g), frontal views, dorsal to the top; (b,f,j,n), dorsal views, anterior to the left; (e,i,l,m), lateral views, anterior to the left.* **(B)** Brunol2, *(a,b), lateral views, animal pole to the top; (c,d,h,l,p), dorsal views, anterior to the left; (f,i), frontal views, dorsal to the top; (g,k,n,o), lateral views, anterior to the left.* **(C)** Brunol3, *(a,c,e,f), lateral views, anterior to the left; (b,g), dorsal views, anterior to the left.* **(D)** Brunol4, *(a), lateral view, anterior to the left; (b), dorsal view, anterior to the left.* **(E)** Brunol5, *(a,b), lateral view, anterior to the left. The positions of the sections were indicated.*

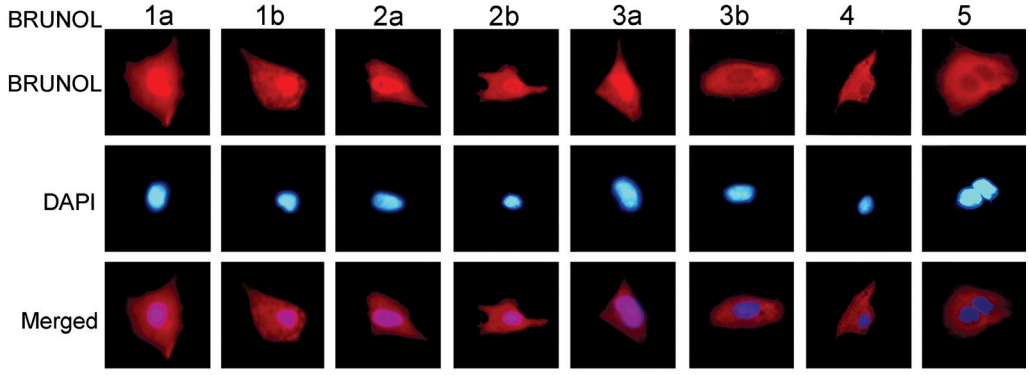


Fig. 4. Subcellular localization of the indicated *Xenopus* BRUNOL proteins. *BRUNOL proteins were detected using anti-FLAG antibody. The cells were also stained with DAPI to visualize the nuclei.*

(Fig. 3Cg,h,i). At stage 36, it was also detected in the facial epibranchial placode (Fig. 3Cf).

The expression of *Bruno4* and *5* were both restricted in the nervous system. *Brunol5* began to express at about stage 25 while *Brunol4* started at stage 30. At stage 30, *Brunol5* was detected in the ventral part of the brain and anterior half of the spinal cord as well as the optic and otic vesicles (Fig. 3Ea). At stage 36, *Brunol4* and *Brunol5* showed overlapping expressing domains in the ventral midbrain, hindbrain, anterior spinal cord, optic and otic vesicles (Fig. 3 D,E). *Brunol5* but not *Brunol4* was detected in the dorsal forebrain and epiphysis (Fig. 3 D,E).

Post-transcriptional regulation exerted by RNA-binding proteins plays important roles in the development and maintenance of the nervous system (reviewed in Perrone-Bizzozero and Bolognani, 2002). For example, the BRUNOL related ELAV/Hu family proteins are also differentially expressed during neurogenesis and are essential for proper neural development (Perron *et al*., 1999; Perrone-Bizzozero and Bolognani, 2002). Our data showed that the *Xenopus Brunol* genes were all strongly expressed in the nervous system, adding more complexity to the post-transcriptional control of neural development. The five *Brunol* genes were all expressed in the neural retinas in the developing eye, with *Brunol2* also strongly in the lens, suggesting an important role of BRUNOLs in eye development.

Subcellular localization of BRUNOL proteins

The full length *Brunol* open reading frames (ORF) were fused with a C-terminal Flag tag coding sequence and the subcellular localization of the BRUNOL proteins was detected using anti-Flag antibody in transfected Hela cells. BRUNOL1a, 1b, 2a, 2b and 3a were all localized in both the cytoplasm and the nucleus, while BRUNOL3b, 4 and 5 distributed only in the cytoplasm (Fig. 4). Interestingly, BRUNOL3a and 3b showed different localization. In the nucleus, BRUNOLs have been shown to regulate the alternative splicing of some pre-mRNAs through binding to a U/G rich sequence element (Barreau *et al.*, 2006). In the cytoplasm, the BRUNOL proteins control the translation or stability of target mRNAs through binding to sequence elements in the 5'- or the 3'-regions (Iakova *et al.*, 2004; Timchenko *et al.*, 1999). The different subcellular localization of the BRUNOL proteins might indicate their different functions.

Materials and Methods

Cloning of Xenopus laevis Brunol *genes and phylogenetic analysis*

Through database searches using mouse *Brunol* mRNA sequences as queries, unigene clusters were identified for *X. laevis Brunol1a* (*Xl*.986), *1b* (*Xl*.12160), *2a* (*Xl*.12254), *2b* (*Xl*.25921) and *3* (Xl.1068). PCR primers were designed according to the EST sequences to amplify the full open reading frames. For *X. laevis Brunol4* and *5*, primers were designed according to the corresponding *X. tropicalis* EST sequences. The primers used were:
Brunol1: forward 5'- TGTTGGGTGGTGGGAGTCTGTTCCTT -3' and reverse 5'- ATGTTCTCCTTGGTCCAGCAGCAGT -3',
Brunol2a: forward 5'- CTGATCAAGAAAACGCTAATGGGGGATA-3' and reverse 5'- ATGGACAAATGGCAGGAAGGGATCAG -3',
Brunol2b: forward 5'- ATAATGAATGGCACAATGGACCACCC -3' and reverse 5'- ATGGACAAATGGCAGGAAGGGATCAG -3',
Brunol3: forward 5'- GCTTCTGTGGCATTGATGTTTGAGC -3' and reverse 5'- CTGGGGTTAGGATCAGTAAGGTTTGC -3',
Brunol4: forward 5'- CAGAGAGACCAGGCAGCTTCCTCT TG -3' and reverse 5'-TCAGTAAGGGCGGTTGGCATCCTTCG -3',
Brunol5: forward 5'- ATGGCCAGACTGACGGAGAGA GAG -3' and reverse 5'- TCAGTATGGTTGGGTTGTGTCCTTAGGC-3'.

cDNAs were reverse transcribed using total RNA from stage 13 to 36 as template and oligo-T 18 as primer (ThermpScript RT-PCR System, Invitrogen). The PCR products were then cloned into pGEM-T vectors (Promega) and the inserts were full length sequenced.

Phylogenetic tree construction was performed as described (Zhao *et al.*, 2007).

RT-PCR assay and restriction enzyme digestion

Total RNAs were extracted using TRIzol regent (Invitrogen) from *X. laevis* embryos at different stages, and the first strand cDNAs were synthesized using random hexamer nucleotide as RT primers (RevertAid H minus first strand cDNA Synthesis kit, Fermentas). The amount of the templates was adjusted according to Histone 4 (H4) expression levels. PCR assays were carried out in the linear phase.
Primers used were:
Brunol1: forward 5'- CGCACAMAGCGCTTACCTCAAT -3' and reverse 5'- GTGGCAGTGAGTGTTGGTGGAG -3',
Brunol2a: forward 5'- TGGGGTAACCTAGCTGGACTCG -3'and reverse 5'- GGGGACTGCTGGAAGAAGTGAG -3',
Brunol2b: forward 5'- TGGGGTAACCTGACTGGACTGA -3' and reverse 5'- GGGGGCTGCTGGAAGAAGTGAG -3',
Brunol3a: forward 5'- GACCATGGAGGGCTGTTCTTCA -3' and reverse 5'- ACCCAGGTTACTGGGGGTGGTA -3',
Brunol3b: forward 5'- GACCATGGAGGGCTGTTCTTCA -3' and reverse 5'- ATGCACCCAGGTTACKGGAGGA -3',
Brunol4: forward 5'- CCAGCTGTGCCTAGCATTCCAT -3' and reverse 5'- GGCTGTGGAAAGGCTTGACTGA -3',
Brunol5: forward 5'- AGACTATGCCTGGGGCCTCATC -3' and reverse 5'- GTAGCTCCCATGCGAAGTGGAC -3'.

PCR conditions: 94°C for 2 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; followed by one cycle of 72°C for 5 min.

To distinguish *Brunol1a* and *b,* the *Brunol1* PCR products were

digested with NcoI before agarose gel electrophoresis, and the software Quantity One (Bio-Rad) was used to analyze the bands' density.

Embryos,* in situ *hybridization and section

In vitro fertilization, embryo culture, and whole mount *in situ* hybridization (WMISH) of *Xenopus* embryos were carried out as described (Zhao *et al.*, 2007). Stained embryos of selected stages were embedded in paraffin, sectioned at 30μm, and photographed using a light microscope (Leica).

Cell transfection and immunohistochemistry

The ORFs of *Brunol* genes were cloned in frame into a eukaryotic expression vector pCS2+ with a Flag tag coding sequence. Cultured Hela cells were then transfected with these vectors using Lipofectamine2000 (Invitrogen) in 96-well plates. At 24h after transfection, cells were fixed with 3.7% formaldehyde for 20min, permeablized in 0.1% Triton X-100/PBS for 10min, washed with PBS, blocked in 1% BSA/PBS for 1h, and then treated with mouse anti-FLAG antibody (Sigma) for 1.5h. After washing with 1% BSA/PBS for 5 times, cells were treated with Cy3-conjugated anti-mouse secondary antibody (Sigma) for 1.5h. The cells were washed and stained with 1μg/ml DAPI (Sigma) for 5 min and then visualized using a fluorescence microscope (Leica).

Acknowledgements

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