



RNA-binding motif protein RBM22 is required for normal development of zebrafish embryos

F. He^{1,2*}, C.-T. Wang^{2*} and L.-T. Gou²

¹College of Life Science, Sichuan University, Chengdu, China

²State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, China

*These authors contributed equally to this study.

Corresponding author: L.-T. Gou

E-mail: goulantu@gmail.com

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ABSTRACT. RBM22 is a newly discovered RNA-binding motif protein, belonging to the SLT11 family; it has been reported to be involved in pre-spliceosome assembly and to interact with the Ca²⁺-signaling protein ALG-2. However, previous studies have not demonstrated whether its expression is essential for early embryogenesis in vertebrates. We utilized zebrafish as a developmental model to study the role of RBM22 during embryogenesis. The aligned protein sequences of RBM22 were found to be highly conserved. *In situ* analysis showed that zRBM22 was expressed as early as the one-cell stage, implying maternal origin during oogenesis; embryos continued to express zRBM22 through at least the 32-h post-fertilization (hpf) stage of development. RT-PCR analysis confirmed the *in situ* expression pattern, indicating that RBM22 is expressed without any spatial and temporal specificity during zebrafish development. zRBM22 morpholino-induced alterations in development were observed as early as 15 hpf of embryogenesis, based on a morphological analysis. Embryogenesis was monitored through 32 hpf; knockdown resulted in a truncated axis and developmental arrest of the head and tail. We conclude that RBM22 plays an important role in zebrafish embryogenesis.

Key words: RNA-binding motif protein; RBM22; Morpholino; Zebrafish; Embryo development

INTRODUCTION

The spliceosome is a large RNA-protein complex, where pre-mRNA splicing proceeds to form the mature mRNA. Five small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6 and a large number of protein splicing factors construct the major spliceosome (Kramer, 1996). Spliceosome assembly follows a carefully orchestrated step-wise pathway (Will and Luhrmann, 2001). It in turn generates the E (early) complex, the pre-spliceosomal A complex and the catalytic C complex (Nilsen, 2000).

Over 300 putative spliceosomal protein components have now been identified by proteomic approaches (Jurica and Moore, 2003); however, many unexpected proteins present, having no apparent direct connection to splicing, still await functional characterization. Some of the additional proteins identified in these complexes have known associations with other aspects of RNA processing, including transcription and mRNA export.

RBM22 is a new RNA-binding motif protein with a conserved RNA-binding domain, a zinc finger of the unusual type C-x8-C-x5-C-x3-H, and a C-terminus that is unusually rich in the amino acids Gly and Pro, including sequences of tetraprolines (Figure 1, Figure 2A). RBM22 belongs to the SLT11 gene family. Slt11p, a splicing factor in yeast, is required for spliceosome assembly. It was identified on the basis of synthetic lethality with a mutation at the 5' end of U2 snRNA where it is involved in intermolecular U2/U6 helix II interaction. The RNA-annealing and -binding activities of Slt11p are two distinct biochemical properties (Xu et al., 1998; Xu and Friesen, 2001). As the homologue of SLT11 in vertebrate, recent studies indicated that RBM22 was involved in pre-spliceosome assembly, functioned in alternative splicing, and was essential for vertebrate development (Chen et al., 2007; Barboric et al., 2009).

To further investigate the role of RBM22 in biological functions, we utilized zebrafish as a developmental model. Using a number of approaches available with zebrafish (Nasevicius and Ekker, 2000), we present data indicating that RBM22 has a functional role during zebrafish embryogenesis.

MATERIAL AND METHODS

Fish and embryo maintenance

Zebrafish (*Danio rerio*), AB strain, were maintained on a 14-h light/10-h dark cycle to stimulate spawning (Westerfield, 1994), and embryos were maintained in Holtfreter's solution at 28.5°C and staged according to Kimmel et al. (1995). Embryos used in whole-mount *in situ* hybridization were raised in 0.003% PTU (Sigma) to prevent pigmentation.

Molecular cloning and sequence analysis

Total RNA was purified with Trizol reagent (Invitrogen) from 36-h post-fertilization (hpf) embryos, and cDNA was synthesized using random primers and reverse transcriptase. 5'-Rapid amplification of cDNA ends (5'-RACE) was carried out using the DD SMART RACE cDNA Amplification Kit according to the manufac-

turer protocol (Clontech). The 5'-RACE fragment of zRBM22 was amplified with the following primer: 5'-ATGGCGACGCCTTTGGGACT-3'. The cDNA fragment was cloned into the pGEM-T Easy vector (Invitrogen).

The nucleotide sequence and predicted amino acid sequence homologies were analyzed via the GenomeNet WWW server using BLASTN and BLASTP searches of the non-redundant database. Phylogenetic analysis was performed using the CLUSTAL W program.

Reverse transcription-polymerase chain reaction (RT-PCR) detection of zRBM22 mRNA

Total RNA from zebrafish embryos at different stages were isolated with the Trizol reagent. cDNA was obtained with the One-Step RT-PCR kit (Takara). β -actin was used as internal control for normalization. β -actin and zRBM22 primers were designed with DNASTar package. β -actin was amplified with the forward primer 5'-ATGGATGATGAAATTGCCGCAC-3' and reverse primer 5'-ACCATCACCAGAGTCCATCACG-3'. zRBM22 primers were forward: 5'-GCGCGCACACCACCGTACTA-3' and reverse: 5'-GGTGGAGGCTCGCATCAGCA-3'.

***In situ* hybridization**

The cDNA fragment of zRBM22 was used to make RNA probes. T3 or T7 RNA polymerase was utilized for the synthesis of either sense or antisense digoxigenin (DIG)-labeled ribonucleotide probes (Ambion, USA).

Whole-mount *in situ* hybridization of zebrafish embryos was performed as previously described (Schulte-Merker et al., 1992). Briefly, embryos at different stages were fixed in 4% paraformaldehyde overnight at 4°C, followed by washing twice with PBS. Embryos were then dehydrated in 100% methanol overnight. Samples were rehydrated followed by a brief incubation with 10 mg/mL proteinase K (incubation time depended on the stage of the embryos) to increase tissue permeability, and the embryos were refixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, hybridization was carried out in 500 mL hybridization buffer (50% formamide, 5X SSC, 500 mg/mL yeast transfer RNA, 0.1% Tween 20, 1% CHAPS and 100 mg/mL heparin) containing 40 ng DIG-labeled RNA probes, at 55°C overnight. After extensive washing, anti-DIG antibodies conjugated with alkaline phosphatase were used to localize mRNA. Color development was carried out using BM-purple under conditions recommended by the manufacturer (Boehringer Mannheim, USA).

Microinjection of antisense morpholino oligonucleotides

Morpholino oligonucleotides were obtained from Gene Tools (LLC, USA). A targeted gene knockdown approach was utilized as reported by Nasevicius and Ekker (2000). The sequence of zRBM22 antisense morpholino was 5'-GTTGGATCCCAAAGACGTCGCCATT-3'. Stock solutions of morpholino oligonucleotides were prepared at 3.0 mM and the procedure for the injection of diluted concentrations followed that described by Nasevicius and Ekker (2000). Individual embryos

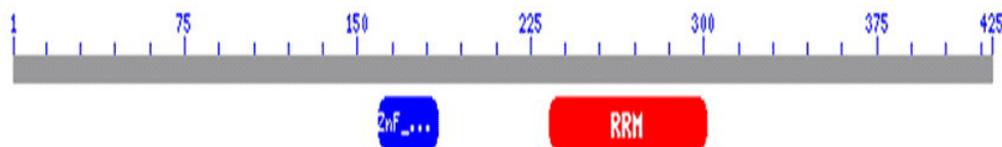
were injected at the 1-cell stage using volumes established by Nasevicius and Ekker using a PLI-90 injector system (Harvard Apparatus).

Control oligonucleotides included scrambled and 4-base mismatch morpholino oligonucleotides. Following injection of various amounts of oligonucleotides, embryos of antisense and control groups were monitored for 15 and 24 hpf, and the number of embryos with an abnormal versus normal phenotype was determined. Images were obtained using a Nikon dissecting microscope.

RESULTS AND DISCUSSION

Cloning and structural analysis of zRBM22

To obtain zebrafish RBM22 cDNA clones, a human RBM22 cDNA clone corresponding to its open reading frame was used to screen a zebrafish oligo dT-primed cDNA library. One full-length zebrafish RBM22 (zRBM22) cDNA clone was obtained from the screening. Analysis of the deduced amino acid sequence of zRBM22 indicated that this open reading frame encodes a protein of 425 amino acids, which contains a conserved RNA-binding domain, a zinc finger of the unusual type C-x8-C-x5-C-x3-H. The C-terminus is unusually rich in the amino acids Gly and Pro, as shown in Figure 1 (obtained from: <http://www.uniprot.org/uniprot/Q6NZZ9>).



Zn-finger

RNA-binding region RNP-1 (RNA recognition motif)

Figure 1. A schematic illustration of zRBM22. Amino acid sequence was deduced from the zRBM22 cDNA. zRBM22 contains a conserved RNA-binding domain and a zinc finger of the unusual type C-x8-C-x5-C-x3-H.

Figure 2A shows the aligned protein sequences of RBM22 of human, monkey, mouse, rat, bovine, frog, and zebrafish. The phylogenetic tree displays that zebrafish and frog share over 90% similarity to RBM22 at the amino acid level with other species. Human, monkey, mouse, rat, and bovine even have 100% similarity to RBM22 (Figure 2B). It is well known that the conservation of a gene in evolution means it has an important biological function. According to evolutionary opinion, the more important a gene is, the more selection pressure it undergoes, and then, the more conserved the gene is. Therefore, the high conservation of RBM22 indicates that it could have an important biological function.

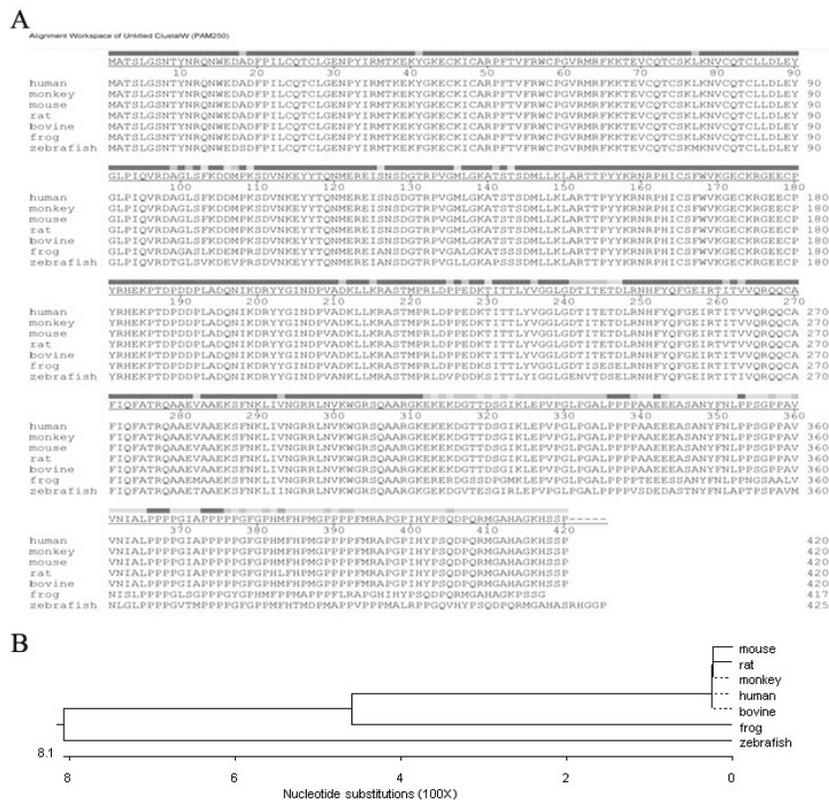


Figure 2. Alignment (A) and phylogenetic tree (B) of the amino acid sequences of RBM22 from various organisms. The amino acid sequences of human, monkey, mouse, rat, bovine, frog, and zebrafish RBM22 were obtained from the GenBank database with accession numbers: Q9NW64, Q4R4J1, Q8BHS3, Q4V7D7, Q3B7L8, Q7ZXB5, and Q6NZZ9, respectively.

Expression pattern of zRBM22 during embryogenesis

To determine the spatial and temporal expression pattern of zRBM22, whole-mount *in situ* analysis was performed. As shown in Figure 3, *in situ* signal for zRBM22 was detected as early as the 1-cell stage (Figure 3A) and continued through the 8-cell stage (Figure 3B), 32-cell stage (Figure 3C), 15-hpf stage (Figure 3D), and 24-hpf stage (Figure 3E). RT-PCR analysis confirmed the presence of zRBM22 mRNA as reflected in the *in situ* studies (Figure 3F) and the amount of zRBM22 mRNA at different stages being nearly equal. The data of RT-PCR and *in situ* analysis at the 1-cell stage showed that RBM22 was a maternal origin gene. Furthermore, RBM22 expressed without any spatial and temporal specificity; that is, it can be detected in different developmental stages and parts of the fish. These results indicated that RBM22 could play an essential role in zebrafish development.

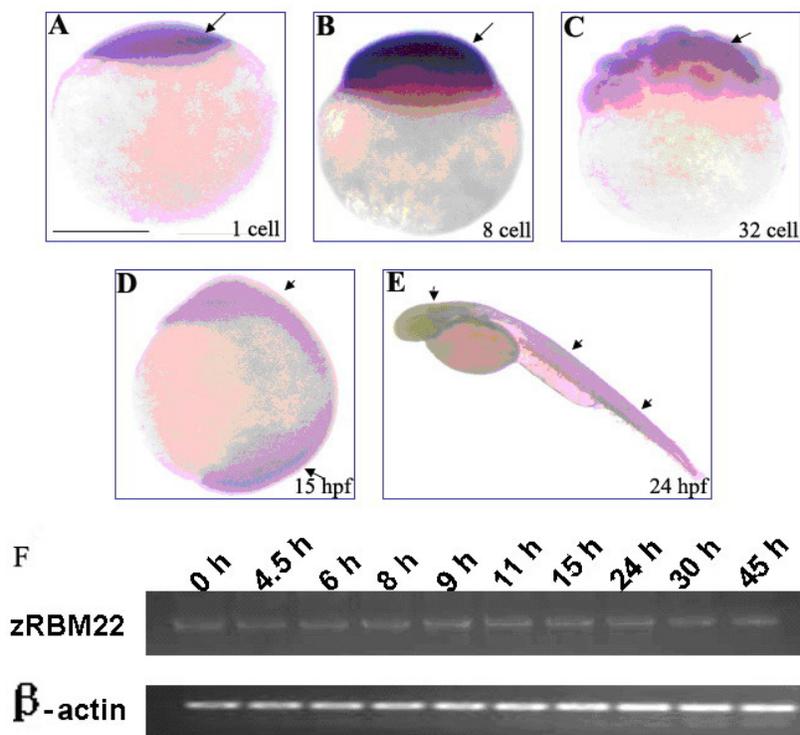


Figure 3. *In situ* analysis and RT-PCR analysis of zRBM22 during zebrafish embryogenesis. zRBM22 mRNA was detected during early stages of zebrafish development. An *in situ* signal was detected as early as the 1-cell stage (A; arrow) and through the 8-cell stage (B; arrow), the 32-cell stage (C; arrow), and the 15-hpf stage (D; arrows). A signal along the entire anterior to posterior axis (arrows) was seen in embryos at 24 hpf (E). RT-PCR analysis of zRBM22 is shown in F. mRNA expression levels for zRBM22 and β -actin (normalizing standard) were determined at 10 stages of development: 1-cell stage, 4.5-h, 6-h, 8-h, 9-h, 11-h, 15-h, 24-h, 30-h, and 45-h stage. hpf = hours post-fertilization. Scale bar equals 250 μ m for A-D and 100 μ m for E.

Loss-of-function analysis of zRBM22

To determine if RBM22 expression is essential for zebrafish development, we performed a loss-of-function analysis using the morpholino oligonucleotide antisense morpholino oligonucleotide, which prevents the translation of zRBM22 mRNA. In the present study, morpholino oligonucleotides were injected into embryos at the 1- to 4-cell stage. As shown in Figure 4, injected morpholino oligonucleotides resulted in an abnormal phenotype with perturbation along the entire anterior-posterior axis and arrested development of the head and tail as viewed from 15 hpf (Figure 4B) through 24 hpf (Figure 4D) and 32 hpf (Figure 4F). However, the mismatch controls had no effect on normal development (Figure 4A, C and E). The results reveal that RBM22 is an indispensable gene in zebrafish embryogenesis.

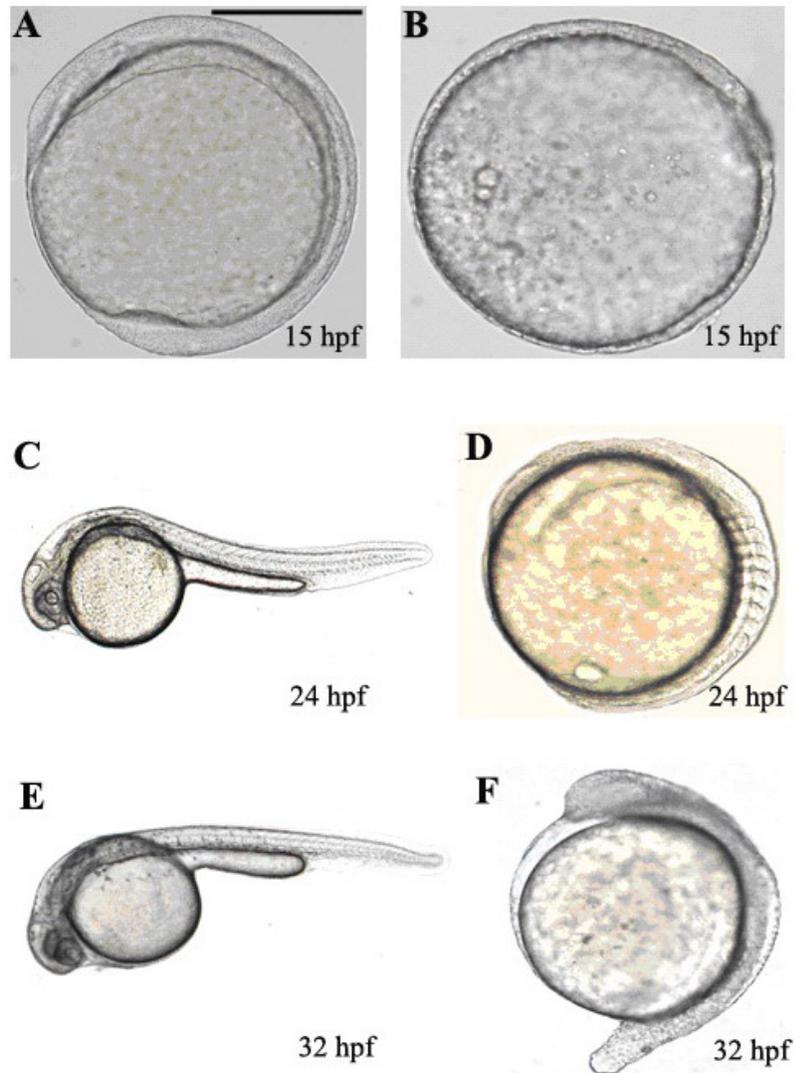


Figure 4. Effects of antisense morpholino oligonucleotides against zRBM22 on zebrafish development. Images of control embryos are shown for the 15-hpf (A), 24-hpf (C) and 32-hpf (E) stages. Embryos with a developmental blocking phenotype following injection of morpholino oligonucleotides to zRBM22 into 1- to 4-cell stage embryos are shown in B (15 hpf), D (24 hpf) and F (32 hpf). hpf = hours post-fertilization. Scale bar equals 250 μ m.

Montaville and co-workers (2006) have reported that increased translation of zRBM22 using an mRNA microinjection approach cannot affect zebrafish early development. Proteomic analysis indicated that as one of the core proteins of a purified human spliceosomal C complex, RBM22 is able to carry out the second step of splicing (Xu and Friesen, 2001;

Bessonov et al., 2008; Barboric et al., 2009). According to over-expression, proteomic analysis and our morpholino oligonucleotide result, we propose that the main function of RBM22 is exclusively to serve as a structural component of the spliceosome. As a structural protein, the overdose RBM22p cannot affect normal cell physiological functions. However, insufficient RBM22p affects the common assembly of the pre-spliceosome and the maturation of pre-mRNA. The consequence in embryogenesis is a block of development

Taken together, zRBM22 is expressed during the earliest stages of zebrafish development, and functional studies indicate that zRBM22 has an important role in the embryogenesis of this vertebrate system.

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