

Involvement of XZFP36L1, an RNA-binding protein, in *Xenopus* neural development

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Abstract: *Xenopus* ZFP36L1 (zinc finger protein 36, C3H type-like 1) belongs to the ZFP36 family of RNA-binding proteins, which contains two characteristic tandem CCCH-type zinc-finger domains. The ZFP36 proteins can bind AU-rich elements in 3' untranslated regions of target mRNAs and promote their turnover. However, the expression and role of ZFP36 genes during neural development in *Xenopus* embryos remains largely unknown. The present study showed that *Xenopus* ZFP36L1 was expressed at the dorsal part of the forebrain, forebrain-midbrain boundary, and midbrain-hindbrain boundary from late neurula stages to tadpole stages of embryonic development. Overexpression of XZFP36L1 in *Xenopus* embryos inhibited neural induction and differentiation, leading to severe neural tube defects. The function of XZFP36L1 requires both its zinc finger and C terminal domains, which also affect its subcellular localization. These results suggest that XZFP36L1 is likely involved in neural development in *Xenopus* and might play an important role in post-transcriptional regulation.

Keywords: ZFP36L1; RNA-binding protein; Neural development; *Xenopus*; Post-transcriptional regulation

mRNA stability is an important mechanism for gene expression regulation, which is critical for embryogenesis and normal physiological processes. The zinc finger protein 36 (ZFP36) family proteins are key players in post-transcriptional regulation and contain two or more highly conserved tandem CCCH zinc fingers (TZF) of tandem Cx8Cx5Cx3H repeats (x represents a variable amino acid). These factors are able to bind to AU-rich elements (AREs) in the 3' untranslated regions (UTR) of targeted mRNAs through their TZFs (Carballo et al, 2000; Lai & Blackshear, 2001; Lai et al, 1999; Lai et al, 2000), which leads to the removal of the poly(A) tail off the targeted mRNAs and promotes their degradation (Carrick et al, 2004; Lai & Blackshear, 2001). The ZFP36 family proteins are evolutionarily conserved, including four rodent members (ZFP36, ZFP36L1, ZFP36L2, and ZFP36L3) and three mammal members (with ZFP36L3 missing, Blackshear et al, 2005), each of which has two conserved ZF domains. Four members have been reported in *Xenopus* and zebrafish, among which ZFP36, ZFP36L1, and ZFP36L2 are conserved while the fourth member (*Xenopus* ZFP36L2.1, ZFP36L2.2

and zebrafish ZFCTH1) contains four ZF domains instead of two. The four ZFP36 genes in *Xenopus* have been cloned and are widely expressed in adult tissues, except for XZFP36L2.1 (XC3H-4), which is detected only in the ovary (De et al, 1999). It has been suggested that XZFP36L2b (XC3H-3b) plays an important role in *Xenopus* kidney development (Kaneko et al, 2003), but little is known about the roles of other ZFP36 members in embryonic development.

Also known as cMG1, TIS11b, ERF1, BRF1, C3H-2, and Berg36 (Barnard et al, 1993; De et al, 1999; Lai et al, 1990; Varnum et al, 1991), ZFP36L1 has been reported to regulate vascular endothelial growth factor (VEGF) mRNA stability in adrenocorticotrophic hormone-stimulated primary adrenocortical cells acting as a negative regulator of VEGF during development

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(Ciais et al, 2004). Research has also shown ZFP36L1 to be regulated by Von Hippel-Lindau (VHL) tumor suppressor protein in renal cancer cells (Sinha et al, 2009). In addition, ZFP36L1 is phosphorylated by p38 MAPK/MAPK-activated protein kinase 2 (MK2) and PKB/AKT pathways. Phosphorylation of ZFP36L1 inhibits its ARE mRNA decay activity, but does not affect its ability to bind AREs (Benjamin et al, 2006; Maitra et al, 2008; Schmidlin et al, 2004). Due to a failure of chorioallantoic fusion, ZFP36L1 knockout mice died in utero before embryonic day 11 (E11) (Stumpo et al, 2004). The XZFP36L1 gene contains 345 amino acids and shows 76% identity to human BRF-1 (De et al, 1999). However, the developmental expression and function of *Xenopus* ZFP36L1 remains largely unknown. In this study, we cloned XZFP36L1 and investigated its expression pattern and potential function during *Xenopus laevis* embryo development.

MATERIALS AND METHODS

Plasmid construction

The XZFP36L1 full open reading frame (ORF) and all truncated fragments were amplified by polymerase chain reaction (PCR) using the XL073b24 clone as a template, which was provided to us by the National Institute for Basic Biology, Japan (NIBB). The PCR primers were:

XZFP36L1 ORF: forward: 5'-ATGTCTACAGCTTTGAT TTCTCC-3'
and reverse: 5'-ATCATCAGAGATAGAAAGTCTGC-3';
N-term truncate: forward: 5'-ATGTCTACAGCTTTGAT TTCTCC-3'
and reverse: 5'-CTGTCCCCCAGGCTTTTGGAGAA-3';
NZF truncate: forward: 5'-ATGTCTACAGCTTTGAT TTCTCC-3'
and reverse: 5'-TCTCTCTTCTGCATTGTGGATG-3';
ZF domains: forward: 5'-CAGGTGAACTCCAGCAGG TACAAG-3'
and reverse: 5'-TCTCTCTTCTGCATTGTGGATG-3';
ZFC truncate: forward: 5'-CAGGTGAACTCCAGCAG GTACAAG-3'
and reverse: 5'-ATCATCAGAGATAGAAAGTCTGC-3';
C-term truncate: forward: 5'-GAGAGACGCTTGGTAT CAGGAC-3'
and reverse: 5'-ATCATCAGAGATAGAAAGTCTGC-3'.

For expression in *Xenopus* embryos and cultured cells, the fragments were cloned into a eukaryotic expression vector pCS2+ with a Flag tag coding sequence at the C-terminal.

Phylogenetic analysis of the ZFP36 protein family

Twenty full coding amino acid sequences of the ZFP36 family were used for Bayesian phylogenetic analysis, including ZFP36_ *H.sapi* (AAH09693),

ZFP36_ *M.musc* (AAH21391), ZFP36_ *X.laevis* (NP_001081884), ZFP36_ *D.rerio* (XP_002665468), ZFP36_ *C.elegans* (NP_505927), ZFP36L1_ *H.sapi* (NP_004917), ZFP36L1_ *M.musc* (NP_031590), ZFP36L1A_ *X.laevis* (NP_001089645), ZFP36L1B_ *X.laevis* (NP_001084214), ZFP36L1A_ *D.rerio* (NP_001070621), ZFP36L1B_ *D.rerio* (NP_955943), ZFP36L2_ *H.sapi* (NP_008818), ZFP36L2_ *M.musc* (NP_001001806), ZFP36L2A_ *X.laevis* (NP_001080610), ZFP36L2B_ *X.laevis* (NP_001081886), ZFP36L2_ *D.rerio* (NP_996938), ZFP36L2.1_ *X.laevis* (NP_001081888), ZFP36L2.2_ *X.laevis* (NP_001108269), ZFP36L3_ *M.musc* (NP_001009549), and ZFCTH1_ *D.rerio* (NP_571014).

Caenorhabditis elegans ZFP36 (C3H-1) were included as an out-group. Sequence alignment was performed using Clustal W (Thompson et al, 1994). The Bayesian approach was implemented in MrBayes3.12 (Ronquist & Huelsenbeck, 2003) with estimation of the substitution rate model, and gamma distribution of among site rate variation two runs were used, each with a single chain of 2×10^7 generations, sampled every 10000 generations. Convergence was assessed by comparing the standard deviation of split frequencies between runs. We excluded 1000 trees from a total sample of 2001 trees in each run. Sample independence was confirmed as no significant increase in log-likelihoods observed after the burn-in phase.

In situ hybridization, section and RT-PCR analysis

Whole mount *in situ* hybridization (WMISH) on *X. laevis* was carried out as previously described (Gawantka et al, 1995). To examine potential overlapping expression, digoxigenin- and fluorescein-labeled RNA probes were used for double WMISH. Plasmid XL073b24 was cut using BamHI, and transcribed with T7 RNA polymerase to generate an antisense probe of XZFP36L1. The probes of *X. laevis* *N-Tubulin* (Papalopulu & Kintner, 1996), *Sox2* (Ma et al, 2007), *Slug* (Aybar et al, 2003), *Xath1* (Kim et al, 1997), *En2* (Hemmati-Brivanlou et al, 1991), *Pax3* (Sato et al, 2005), *Dbx2* (Ma et al, 2011), and *Nkx6.2* (Zhao et al, 2007) were used as previously described. Stained embryos were embedded in paraffin, sectioned at 20 μ m, and photographed using a light microscope (Leica).

Semi-quantitative reverse transcription PCR was carried out as previously described (Wu et al, 2010). The PCR primers and conditions were:

XZFP36L1A: forward: 5'-TGCATGAGCAGCAGACGT G-3' and reverse: 5'-GGTCTTATGCGTCTCTCCATG-3', 30 cycles;

XZFP36L1B: forward: 5'-TGCATGAGCAGCAGAGGA A-3' and reverse: 5'-GCCCTTCTGTGTCTCTAAACA-3', 30 cycles; *H4* was used as a loading control: forward: 5'-CGGGA TAACATTCAGGGTA-3' and reverse: 5'-TCCATGGCG GTAACGTGTC-3', 26 cycles.

Embryo culture and microinjection

In vitro fertilization, embryo culture, and WMISH of *Xenopus* embryos were carried out as per Gawantka et al (1995). Embryos were staged according to Nieuwkoop and Faber (1967). Capped mRNA of *X. laevis* ZFP36L1 and its truncated forms were synthesized *in vitro* using SP6 mMessage Machine kit (Ambion). Microinjection was carried out using the PLI-1 Pico-injector (Harvard Apparatus) equipped with an MK-1 micromanipulator (Singer Instruments). Embryos were injected in one dorsal blastomere at the 4-cell stage with 0.5–1 ng XZFP36L1 mRNA or 0.3–0.6 ng of truncated mRNA. Injected mRNA was traced by co-injected 100 pg nuclear β -galactosidase, which was stained with Red-Gal substrate (Research Organics). The detection of β -galactosidase activity for tracing cell lineage was carried out as previously described (Kurata & Ueno, 2003).

Cell transfection and immunohistochemistry

The XZFP36L1 ORF and its truncated constructs were cloned in frame into a eukaryotic expression vector pCS2+ with a C-terminal Flag tag coding sequence. Hela cell culture, transfection, and immunohistochemistry were carried out as described in previous research (Wu et al, 2010).

RESULTS

Cloning and phylogenetic analysis of ZFP36 family proteins

Through BLAST search against EST databases, two alleles of *X. laevis* ZFP36L1 were found, XZFP36L1A

and XZFP36L1B, which shared 97% identity at the amino acid level. The full ORF of XZFP36L1B was cloned by PCR using an EST clone as a template (XL073b24, from the National Institute for Basic Biology, Japan). In the following studies on XZFP36L, this allele was used unless otherwise stated. Consistent with its mammal orthologues, XZFP36L1 had two tandem ZF domains. To confirm the evolutionary relationships between the homologues of ZFP36L1, a phylogenetic tree was constructed using MrBayes. The nomenclature of the XZFP36 members was according to Xenbase (Bowes et al, 2008). The ZFP36 family proteins were grouped into four well-supported vertebrate clades inferred from the phylogenetic results (Figure 1). The results indicate that ZFP36L1, ZFP36L2, and ZFP36L3 were indeed paralogues that arose by gene duplication from a common ancestor to the vertebrate lineage. However, ZFP36L3 in rodents and the fourth ZFP36 genes in fish (ZFCTH1) and frog (ZFP36L2.1 and 2.2) were lineage specific. Among the ZFP36 related proteins, members of the ZFP36L1 and ZFP36L2 subfamilies were more closely related.

Temporal and spatial expression of XZFP36L1 during *Xenopus laevis* early development

We examined XZFP36L1 expression during *X. laevis* embryogenesis by RT-PCR and WMISH. Both XZFP36L1A and B showed similar temporal expression patterns, as detected by RT-PCR. They were both expressed at low levels maternally and in embryos from blastula to gastrula stages. Their expression went up at early neurula stages and remained stable at later stages (Figure 2A).

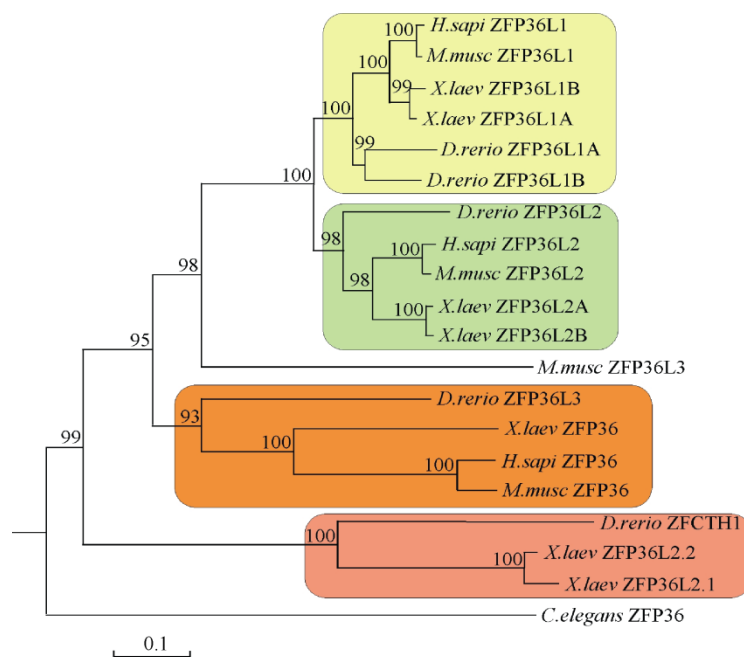


Figure 1 Bayesian tree depicting the phylogenetic relationships of the ZFP36 proteins. Branch confidence values are shown as Bayesian posterior probabilities. The scale bar shows the number of substitutions per site.

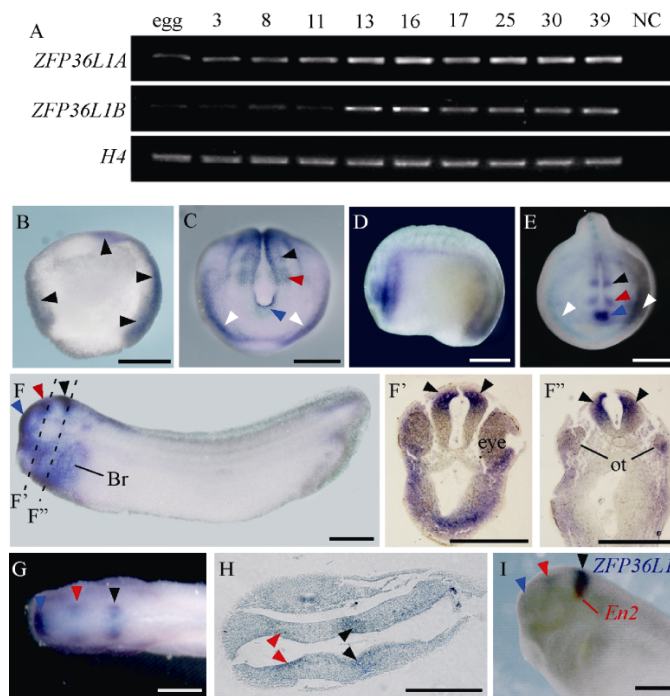


Figure 2 Temporal and spatial expression patterns of XZFP36L1

A: RT-PCR analysis of the developmental expression of XZFP36L1A and B; B–I: Expression patterns of XZFP36L1 revealed by WMISH and paraffin sections; B: Stage 10, the embryo was bisected, the arrowheads indicate the expression of XZFP36L1 in the ectoderm and involuting mesoderm; C: Stage 16, anterior view; D: Stage 20, lateral view; E: Stage 20, anterior view; F–I: Stage 32 embryos: F: Lateral view. The broken lines indicate the position of corresponding sections showed in F' and F''. Br, branchial arches. F', F'': Transverse sections of stage 32 embryo, the arrowheads indicate the expression of XZFP36L1 in the dorsal brain. ot, otic vesicle; G: Dorsal view of the head region; H: Horizontal section showing its expression in the fore-midbrain boundary (red arrowheads) and mid-hindbrain boundary (black arrowheads); I: Double WMISH of *En2* (red) and XZFP36L1 (blue). Arrowheads in (C–I) indicate the expression of XZFP36L1 in the forebrain (blue), fore-midbrain boundary (red) and mid-hindbrain boundary (black). White arrowheads in (C) and (E) indicate its expression at the presumptive neural placode region. Bars in (B–I)=0.5 mm.

Since the two alleles of XZFP36L1 shared more than 90% identity, it was difficult to design probes to distinguish their expression patterns by *in situ* hybridization. Here we used XZFP36L1B as a template for *in situ* probe. At the gastrula stage, XZFP36L1 transcripts were detected in both ectoderm and mesoderm (Figure 2B). During neurula stages, its expression became enriched in the neural system, especially in the anterior neural folds (Figure 2C), where their expression became gradually restricted into three discontinuous patches, corresponding to the presumptive forebrain, fore-midbrain boundary, and mid-hindbrain boundary, respectively (Figure 2D, E). Its expression was also detected in the placode regions at the anterior neural plate borders (Figure 2C, E). At tadpole stages, XZFP36L1 remained expressed in the three discontinuous regions of the brain and also in the branchial arches (Figure 2F, G). These domains were confirmed to be the forebrain, fore-midbrain boundary, and mid-hindbrain boundary, respectively, by horizontal sections (Figure 2H, arrow heads) and double *in situ* hybridization with *En2*, a mid-hindbrain boundary marker (Figure 2I). Transverse sections of stage 32 embryos showed that XZFP36L1 was mainly expressed

at the dorsal part of the neural tube (Figure 2F', F''). These data showed that XZFP36L1 was expressed in specific brain regions during neurulation and might play a role in neural development.

Overexpression of XZFP36L1 leads to neural tube defects

To investigate the function of XZFP36L1 during *Xenopus* embryogenesis, we overexpressed XZFP36L1 in *Xenopus* embryos by injecting synthetic XZFP36L1 mRNA into one dorsal cell at the 4-cell stage. Interestingly, the injected embryos showed severe neural tube defects (NTDs). Compared to the control group, the neural tubes of the injected embryos failed to close properly and remained open at tailbud stages (57%, $n=81$, Figure 3A, B). To further investigate the role of XZFP36L1 in neural development, we checked the expression of several neural markers in XZFP36L1 mRNA injected embryos. The results showed that many of the neural markers were inhibited at neurula and tailbud stages, including *N-tubulin* (67%, $n=24$, Figure 3C), *Sox2* (86%, $n=35$, Figure 3D), *Slug* (85%, $n=20$, Figure 3E), *En2* (84%, $n=25$, Figure 3F), *Nkx6.2* (73%, $n=15$, Figure 3G), *Pax3* (70%, $n=17$, Figure 3H), *Dbx1* (69%, $n=16$, Figure 3I), and *Xath1* (62%, $n=13$, Figure 3J).

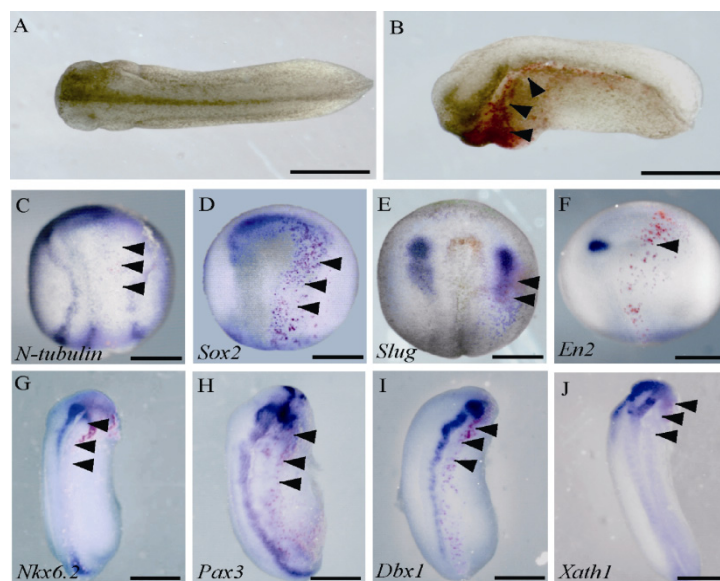


Figure 3 Overexpression of XZFP36L1 leads to NTDs in *Xenopus* embryos

A: Control embryos, stage 25; B: Embryo injected with XZFP36L1 mRNA shows severe defects during neural tube closure (arrowheads), stage 25; C–J: Overexpression of XZFP36L1 inhibits neural differentiation. Expression of neural marker genes *N-tubulin* (C), *Sox2* (D), *Slug* (E), *En2* (F), *Nkx6.2* (G), *Pax3* (H), *Dbx1* (I) and *Xath1* (J) is largely inhibited in the injected sides (arrowheads). (C–F), stage 15; (G–J), stage 25. Bars=0.5 mm.

These results suggested that overexpression of XZFP36L1 inhibited neural induction and differentiation in *Xenopus* embryos, leading to severe neural tube defects.

The ZF and C-terminal domains of XZFP36L1 are required for its activity and subcellular localization

The XZFP36L1 protein contains an N-terminal domain, two ZF domains, and a C-terminal domain (Figure 4A). To determine which domains are essential for its function in neural development, several truncated forms of XZFP36L1 were constructed (Figure 4A), transcribed into mRNAs, and injected into *Xenopus* embryos. Embryos were examined at stage 20, when the neural tube was completely closed. The results showed that truncates containing both the ZF and the C-terminal domains led to defects in neural tube closure for *Xenopus* embryos like the full length ORF (Figure 4F), whereas other truncated forms lacking either the ZF motif or the C-terminal domain did not exhibit such an effect (Figure 4D, E, G). These results suggest that both the ZF and the C-terminal domains were necessary for its function.

The XZFP36L1 gene belongs to the ZFP36 RNA-binding protein family. The ZF domains of ZFP36 family proteins are suggested to bind to the AREs of targeted 3' UTRs. Thus, these proteins are supposed to locate in the cytoplasm to execute their functions. To determine whether the activity of XZFP36L1 in neural development was related to its subcellular localization, we fused the full-length XZFP36L1 ORF and its truncates with a C-terminal Flag tag coding sequence and performed immunohistochemistry using anti-Flag antibodies in

transfected Hela cells. As expected, the full length ORF was detected dominantly in the cytoplasm, which was consistent with human ZFP36L1 (CMG1) results (Figure 4H; Phillips et al, 2002). The construct containing only the N-terminal domain showed granular distribution in the cytoplasm (Figure 4I). All the other truncated forms were more enriched in the nucleus, except for the one containing both the ZF and C-terminal domains, which showed higher signal in the cytoplasm compared to other truncates (Figure 4J–M). According to the above data, the activity of these truncates in NTDs seemed consistent with their localization in the cytoplasm, indicating that the cytoplasmic localization of ZFP36L1 was important for its function.

DISCUSSION

In this study, we showed that XZFP36L1 was expressed in specific brain regions in *Xenopus* embryos, and overexpression of this protein inhibited neural induction and differentiation and led to severe NTDs, indicating that XZFP36L1 was involved in *Xenopus* neural development. We also tried to knockdown the expression of endogenous ZFP36L1 using specific morpholinos. Unfortunately, no clear phenotypes were observed in the injected embryos, although the morpholinos actively inhibited the expression of the reporter GFP genes carrying the targeted sequences (data not shown). The reason for this might be that either the morpholino was not efficient enough to block XZFP36L1 expression, or its function was compensated by other members of this gene family, since it has been

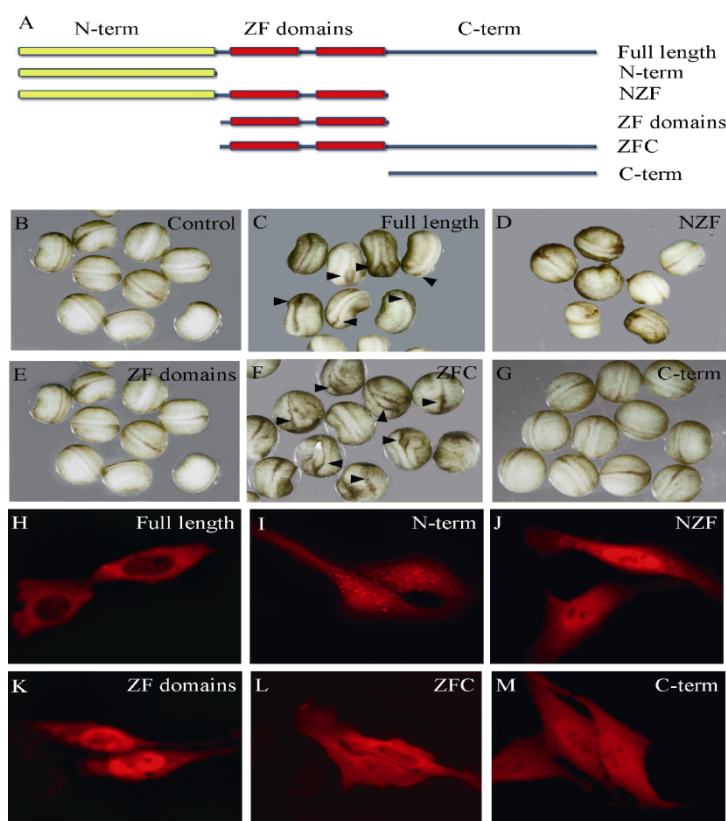


Figure 4 Activity and cellular localization of different XZFP36L1 truncates

A: The schematic structures of XZFP36L1 protein and its truncates; B-G: Both the ZF motif and the C-terminal domain are required for its activity in neural development; B: control embryos. Overexpression of full length XZFP36L1 (C) or the ZFC truncate (F) leads to defects in neural tube closure (arrowheads), while overexpression of the NZF truncate (D) or ZF domains (E) or C-terminal truncate (G) has no obvious effect on neural tube closure; H-M: Localization of XZFP36L1 and its truncates in transfected HeLa cells.

reported that the ZFP36 protein family are all RNA-binding proteins and may share some common target mRNAs, *e.g.*, TNF- α (Carballo et al, 1998; Lai et al, 2000), GM-CSF (Carballo et al, 2000; Lai & Blackshear, 2001), and VEGF (Bell et al, 2006; Brennan et al, 2009; Ciaia et al, 2004).

The function of XZFP36L1 required both its ZF and C terminal domains, the ZF domains for binding to AREs and targeting mRNA degradation and the C-terminal domain to contain nucleus export sequences responsible

for cytoplasmic localization (Phillips et al, 2002). Our results indicated that the cytoplasmic localization of ZFP36L1 was important for its function, and suggested that ZFP36L1 was involved in neural development, likely by acting as an RNA-binding protein. However, the *in vivo* function of XZFP36L1 during brain development requires further detailed analysis.

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