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Biochemical and Biophysical Research Communications 306 (2003) 1106–1111
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A novel TBP-interacting zinc finger protein functions in early development of *Xenopus laevis*

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Received 12 May 2003

Abstract

A zinc finger protein that interacts with *Xenopus* TATA-binding protein was previously isolated by a yeast two-hybrid screen and found to serve as a transcriptional repressor. The gene was designated the negatively regulating zinc finger protein gene (*NZFP*). Herein, *NZFP* was found to be expressed maternally. After gastrulation, the level of *NZFP* mRNA decreased significantly throughout the neurula stage. However, mRNA levels increased at stage 35 and then began to decrease at stage 48. Eventually, no *NZFP* mRNA was observed in adult tissues except in the ovary. *NZFP* mRNA was detected in the animal hemisphere during gastrulation and observed in the neural ectoderm at the neurula stage. At the tailbud stage, *NZFP* was highly expressed in the head tissues such as brain, eyes, otic vesicles, lateral line placodes, and branchial arches, but weakly in somites. Depletion of *NZFP* in the embryos using RNA interference caused premature death at the gastrula stage or induced secondary partial axis after gastrulation. These results strongly suggest that NZFP is an essential transcription factor involved in the cell movement during gastrulation and the formation of the dorsal axis during early development in *Xenopus*.

Keywords: Embryogenesis; FAX; Development; *Xenopus laevis*; Zinc finger protein; RNA interference

We have been investigating the mechanism of selenocysteine tRNA gene transcription in *Xenopus laevis* [1]. In previous studies, we obtained evidence that a novel transcription factor interacts with the TATA-binding protein (TBP) to regulate specific types of genes in *X. laevis* [2]. We employed a yeast two-hybrid screening system to isolate this transcription factor. As a result, seven positive genes were isolated, one of which showed high homology to XLcGF53.1 that is a member of the finger-associated box-containing zinc finger protein (FAX-ZFP) subfamily [3]. The biological roles of most FAX-ZFPs have not yet been determined. In fact, the apparent molecular function of the FAX domain remains to be assessed [4]. These FAX-ZFPs contain conserved sequence elements associated with the zinc finger module named the FAX domain. The FAX domain is composed of multiple unit sequences occurring in different combinations for the individual members of the *Xenopus* ZFP family [3]. In our recent studies, we found that this gene could serve as a transcriptional repressor and the FAX domain is required for repression [5]. We, therefore, designated this gene the negatively regulating zinc finger protein gene (*NZFP*).

Herein, we describe the expression pattern of *NZFP* and elucidate its function during *Xenopus* embryogenesis. The maternal expression of *NZFP* was restricted to the animal hemisphere, and the zygotic expression was detected in the neuroectoderm, and enriched in the head

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)01069-6

tissues at the tadpole stages. To elucidate the role of NZFP in development, RNA interference (RNAi) experiments using double-stranded RNA were performed. The depletion of *NZFP* mRNA by RNAi caused premature death during gastrulation and induced secondary partial axis among the surviving embryos.

Materials and methods

RNA preparation. The template plasmid (*pBS-ZF*) into which the zinc finger domain of *NZFP* ORF was subcloned was digested with *Bam*HI and *Not*I. Both sense and antisense RNA were synthesized in vitro with T3 and T7 RNA polymerase, respectively. Double-stranded RNA (dsRNA) for the interference experiment (RNAi) was prepared by annealing sense and antisense RNA [6,7].

Xenopus embryos and microinjection. Xenopus embryos were obtained from in vitro fertilized wild type eggs, grown and microinjected according to the standard procedures described by Godsave et al. [8]. Embryos were staged according to Niuwkoop and Faber [9]. Two/four-cell stage embryos were injected in the equatorial region with 0.5, 1.0, and 3.0 ng of dsRNA [10].

Whole-mount in situ hybridization. Embryos at various stages were fixed in MEMFA (100 mM Mops, 2 mM EDTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 2 h and transferred to 100% methanol. Whole-mount in situ hybridization was performed as described [11]. To prepare the probe, the template plasmid (*pBS-ZF*) was linearized with *Bam*HI, and antisense RNA was synthesized and labeled in vitro with T3 RNA polymerase by using digoxigenin (DIG)-UTP. Hybridization was detected with alkaline-phosphatase coupled anti-DIG antibody and visualized using BM purple (Roche Molecular Biochemicals) as substrate. Stained embryos were embedded in paraffin and sectioned into 12 μm thick slices [12].

Northern blot analysis. For Northern blot analysis, RNAs were extracted from unfertilized eggs, diverse stages of embryos, and several tissues of adult *Xenopus*. The RNAs were subjected to 1% formaldehyde–agarose gel electrophoresis after loading 10 μg of total RNA in each lane and transferred onto Hybond–N membrane (Amersham–Pharmacia Biotech) from the developed gels. The probe that corresponds to the C-terminus half of *NZFP* was hybridized overnight to the blot in the hybridization solution (5× SSC, 0.5% SDS, 3× Denhardt's solution, and 50% formamide) at 42 °C.

RT-PCR. Embryos were collected at stages 11 and 22. Total RNA was isolated from embryos using TRIZOL Reagent (Invitrogen) and was used for cDNA synthesis. Reverse transcription (RT) was performed using oligo (dT) primers, total RNA (5 μg), and Superscript II reverse transcriptase (Life Technology) [13,14]. Two microliters of reaction mixture containing synthesized cDNA was used for the subsequent PCR. PCR primers for the 3′-untranslated region of *NZFP* mRNA were as follows: forward, 5′-AGTGGACTGCTTAAAGAG-3′ and reverse, 5′-GGACATGGATTAAAATCA-3′. RT-PCR was also performed with EF1α primers as an internal control.

Results and discussion

Comparison of NZFP with the XLcGF53.1 protein

The deduced amino acid sequence of *NZFP* cDNA (GenBank Accession No. AY211520) showed significantly high homology to XLcGF53.1 (GenBank Accession No. M25872). However, it was found that the length of the XLcGF53.1 protein was 97 amino acids shorter than NZFP which contains 613 amino acids. When both sequences were aligned from their N-termini and their overlapping, 516 amino acids compared, NZFP shares 97.8% identity with XLcGF53.1 (Fig. 1). As shown in the alignment, 11 amino acid residues were not identical in the N-terminal FAX domains, but all residues were identical in the C-terminal zinc finger domains. The sequence homology between NZFP and XLcGF53.1 is higher than any other homology comparisons between other FAX-ZFP members. Based on this fact, *NZFP* may virtually be the full length cDNA of XLcGF53.1. XLcGF53.1 has been reported to be expressed during early developmental stages, but its expression was limited to the somite stage [3]. To elucidate the possible biological role of NZFP, we analyzed its detailed expression pattern in a broad range of developmental stages, including spatiotemporal and tissue specific expression as described below.

NZFP expression during early Xenopus development

The Northern blot analysis showed that *NZFP* mRNA was expressed maternally (Figs. 2A and B), and interestingly, it was also induced zygotically during late stages of embryogenesis (Fig. 2A). The maternal *NZFP* mRNA was abundant in the mature oocyte and egg. After gastrulation, the level of *NZFP* mRNA, however, decreased significantly between stages 12 and 32. *NZFP* expression was increased at stage 35 and then began to decrease at stage 48. The relative changes in *NZFP* mRNA levels during development were compared with those of the ubiquitously expressed *histone H4* gene which was used as a control (Fig. 2A). Eventually, no *NZFP* mRNA was observed in adult tissues except in the ovary (Fig. 2B).

For a more detailed analysis of *NZFP* expression patterns, whole-mount in situ hybridization was carried out with embryos at various stages. The majority of *NZFP* mRNA was localized to the animal pole in the blastula stages and it was hardly detectable in the vegetal pole (Figs. 3A and B). The *NZFP* mRNA was detected in the animal hemisphere and excluded from the vegetal hemisphere at the early gastrula stage (Fig. 3C). A sagittal section of the embryo at the early gastrula stage showed a high level of *NZFP* expression in the ectoderm of the animal hemisphere (Fig. 3D). During neurulation, *NZFP* mRNA was detected at a high level in the neural ectoderm and the midline excluding its expression in the neural fold region at the early neurula stage (Fig. 3E). At the late neurula stage, *NZFP* mRNAs were localized in the sensorial layer of ectoderm (Figs. 3F and G). At the early tailbud stage, *NZFP* expression was observed in an anterior neural tube including the head region (Fig. 3H). At the tadpole stage, *NZFP* mRNA was detected primarily in the head region

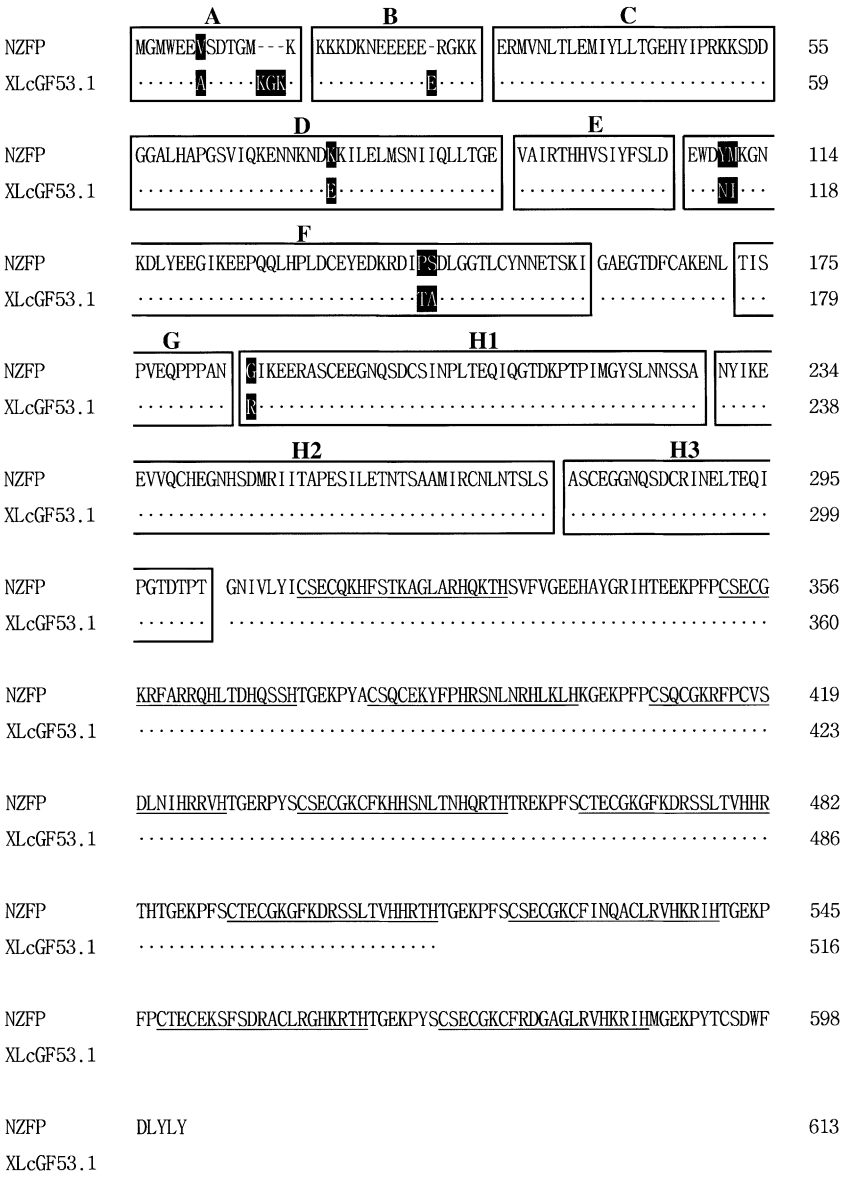

Fig. 1. Comparison of the *Xenopus* NZFP amino acid sequence with that of XLcGF53.1. The sequence of XLcGF53.1 was taken from GenBank. Dots indicate identical residues. Dashes represent gaps introduced to maximize the alignment and unmatched amino acids are shaded in black. Each amino acid block of the FAX domain (A–H3) is boxed and zinc finger motifs are underlined.

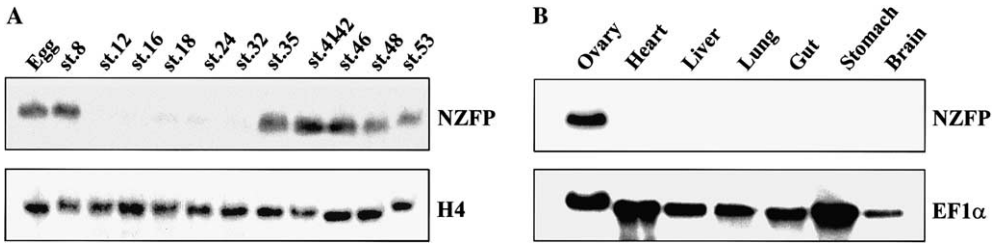

Fig. 2. Northern blot analysis of *NZFP* expression. (A) Expression during *Xenopus* development. RNAs were extracted at the developmental stages shown, electrophoresed, transferred to filters, the filters hybridized with probe and autoradiographed as given under Materials and methods. *Histone H4* gene, which is ubiquitously expressed, was used as a control. (B) Tissue specificity of expression. RNAs were extracted from indicated *Xenopus* adult tissues. EF1α was used as an internal control.

such as brain, eyes, otic vesicles, lateral line placodes, and branchial arches and weakly in somites (Figs. 3I and J). Transverse sections of tadpole stage embryos also revealed that *NZFP* expression was localized to the brain, eyes, and head mesenchyme, and weakly in the neural tube and notochord (Figs. 3K and L). This result

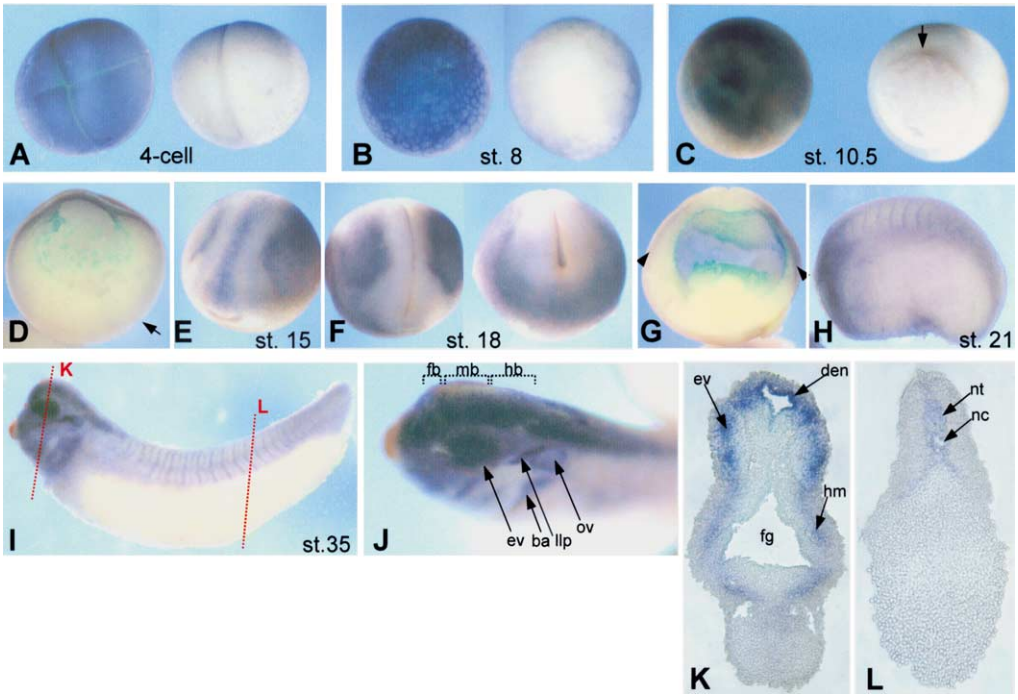

Fig. 3. Spatiotemporal expression of *NZFP* during *Xenopus* development. Whole-mount in situ hybridization was performed with *NZFP* antisense RNA and staged *Xenopus* embryos. (A) Animal/vegetal view of four-cell stage; (B) animal/vegetal view of stage 8; (C) animal/vegetal view of stage 10.5, arrows in (C) and (D) indicate dorsal lip of blastopore; (D) sagittal section of stage 10.5; (E) dorsoanterior view of stage 15; (F) dorsoanterior/anterior view of stage 18; (G) transverse section of stage 18, arrowheads indicate the sensorial layer of ectoderm; (H) stage 21, anterior to the left; (I) lateral view of stage 35; (J) head region in (I); and (K, L) transverse sections as indicated by dashed lines across the embryo in (I). EF1α was used as an internal control. *Abbreviations:* anc, anterior neural crest; ba, branchial arches; den, diencephalons; ev, eye vesicle; fb, forebrain; fg, foregut; hb, hindbrain; hm, head mesenchyme; llp, lateral line placodes; mb, midbrain; nc, notochord; nt, neural tube; and ov, otic vesicle.

suggests that *NZFP* expression is tightly regulated both temporally and spatially in early development.

Based on the temporal expression pattern of *NZFP*, the amount of *NZFP* mRNA was decreased to its lowest level during neurulation. These Northern hybridization results were consistent with the weak and limited staining of *NZFP* mRNA in whole-mount in situ hybridization during neurulation. It should be noted that NZFP is a transcriptional repressor and although the relative amount of *NZFP* mRNA was reduced in the neurula stages, it was confined to the neuroectodermal tissues. Our result also showed that *NZFP* expression is reactivated zygotically at the late stage of embryogenesis. Knöchel et al. [3] reported that XLcGF53.1 is expressed only in the early stages of *Xenopus* development. The inconsistency between our result and theirs seems to be derived from the range of stages examined. XLcGF53.1 mRNA was not detected in the neurula and somite stages [3]. Our results show that *NZFP* was minimally expressed during stages 12–32 which corresponded to the neurula and somite stages. It seems that Knöchel et al. [3] did not examine embryos at later developmental stages. This zygotic expression in later stages of embryogenesis is widespread within the tissues in the head. However, it still remains to be determined whether the promoters or genes that regulate maternal and zygotic expression are identical.

Depletion of maternally expressed NZFP causes premature death or axis duplication

To define the function of NZFP during early development in *Xenopus*, we performed RNAi experiments by using double-stranded RNA (dsRNA) corresponding to *NZFP*. 0.5, 1.0, and 3.0 ng of dsRNA of *NZFP* (dsNZFP) were injected into the equatorial region of 2/4-cell stage embryos, respectively. Control and injected embryos were cultured until the equivalent of tadpole stage 35 and monitored for their phenotypic appearance.

The results of dsRNA microinjection experiments are summarized in Table 1. The injection of <0.5 ng of dsNZFP per embryo caused little effect on embryo survival or abnormality, when compared with the embryos injected with dsRNA encoding the green fluorescence protein (GFP) as a negative control (dsGFP). Significant developmental abnormalities, however, appeared when dsNZFP was injected into embryos at a dose of 1 ng. Closure of the blastopore of these embryos was delayed approximately twofold compared to controls (Figs. 4A

Table 1
Effects of dsRNA injection on embryogenesis

RNA injected* (amount)	Survived (%)	Normal**	Secondary partial axis	Dead during gastrulation	Other abnormal
dsNZFP (0.5 ng)	76.2	66.7	2.4	23.8	7.1
dsNZFP (1 ng)	58.2	41.8	16.4	41.8	0
dsNZFP (3 ng)	40	12.5	5	60	27
dsGFP (3 ng)	98	98	0	0	2

* Total number of each group was at least 50 embryos.
** Phenotypes were observed at stage 35.

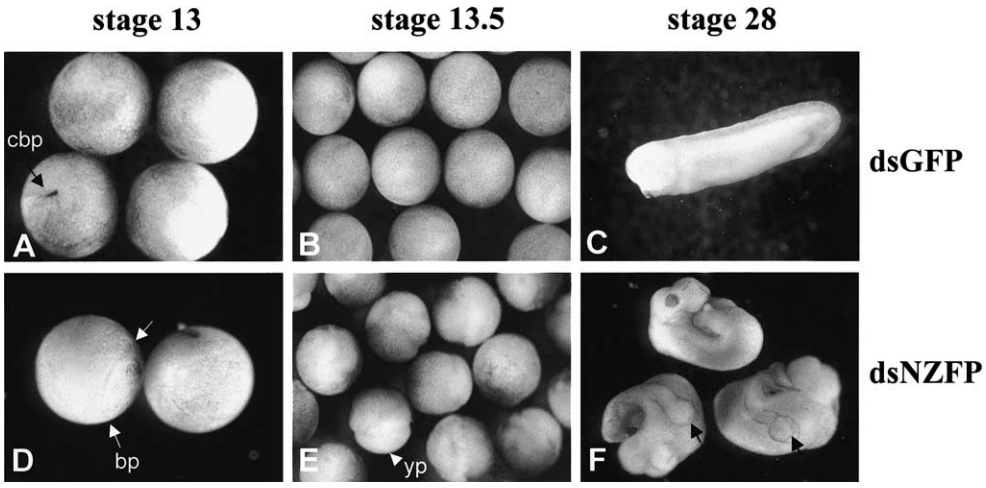

Fig. 4. Effects of *NZFP* dsRNA (dsNZFP) injection on embryogenesis. dsRNA was injected into the equatorial regions of two blastomeres at the two- or four-cell stage. *GFP* dsRNA (dsGFP) was injected into embryos as a control (A–C). Panels A and D show stage 13, panels B and E, stage 13.5, panels C and F, stage 28, and panel F shows abnormal phenotype including dual axis and defects of neural fold closure that survived after injection (black arrows indicate secondary partial axis). Black arrow indicates closed blastopore (cbp) in panel A. In panels D and E, white arrows indicate opened blastopore (bp) and arrowhead indicates yolk plug (yp).

and D). When they were allowed to develop further, 41.8% of the embryos died at the midgastrula (Table 1; Fig. 4E) and secondary partial axis was induced in 16.4% of the embryos (Table 1; Fig. 4F). In addition, the injection of 3 ng of dsNZFP increased the embryonic death rate during gastrulation to 60%, while the ratio of normally developed embryos and those containing secondary partial axis decreased to 12.5% and 5%, respectively. It should be noted that the remaining 27% of the injected embryos showed an abnormal phenotype including short axis and defects of neural fold closure (Table 1). The injection of 3 ng of dsGFP showed no effect on development (Figs. 4A and B).

RT-PCR was performed to examine whether the endogenous *NZFP* mRNA was actually reduced by dsRNA injection. The primers for RT-PCR were designed in the 3′ untranslated region of the *NZFP* mRNA. Fig. 5 showed that the level of *NZFP* mRNA in dsNZFP injected embryos was reduced by at least fourfold more than that in dsGFP injected embryos when normalized with the levels EF1α mRNA as a control.

It was clearly shown that the depletion of *NZFP* by RNAi caused premature death during gastrulation and dual axis formation during neurulation. Northern blot results showing an elevated level of *NZFP* before late gastrula stage support the idea that NZFP may be involved in the cell movement during gastrulation. Also, it is interesting to note that the depletion of *NZFP*, which is highly expressed in the head including brain, eyes, and

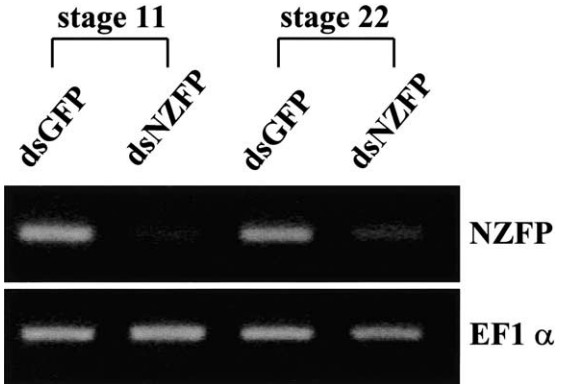

Fig. 5. Reduction of the endogenous *NZFP* mRNA by dsNZFP injection. Embryos were injected with double-stranded RNA (dsRNAs) as indicated. RT-PCR was carried out with *NZFP*-specific primers using total RNA isolated from stage 11 and stage 22 embryos. EF1α was used as an internal control.

otic vesicles, induced the secondary head-like partial axis. These studies propose that NZFP is involved in head or axis formation. The results presented herein, and our other study that elucidates NZFP as a negatively regulating transcription factor [5], suggest that NZFP may play a role as an essential transcription factor required for early *Xenopus* development.

Acknowledgments

This work was supported by 21C Frontier Functional Human Genome Project from the Ministry of Science and Technology of Korea and Korea Research Foundation Grant (KRF-2001-015-DS0066) to B.J.L. and by BK21 Research Fellowship from the Ministry of Education and Human Resources Development to M. Kim and J. Choi.

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