

## Transcriptional regulation of *Zic3* by heterodimeric AP-1(c-Jun/c-Fos) during *Xenopus* development

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Abbreviations: AP-1, activator protein-1; *Zic3*, zinc finger protein of the cerebellum

### Abstract

The heterodimeric c-Jun/c-Fos, an activator protein-1 (AP-1) has been implicated in mesoderm induction (Dong *et al.*, 1996; Kim *et al.*, 1998) whereas the homodimer of c-Jun was reported to be involved in neural inhibition during the early development of *Xenopus* embryos. During the early vertebrate development AP-1 involvement in the neural induction is still not clearly understood. We report here that AP-1 has a role in *Zic3* expression, a critical proneural gene and a primary regulator of neural and neural crest development (Nakata *et al.*, 1997; Nakata *et al.*, 1998). AP-1 was able to induce the *Zic3* gene in a dose dependent manner but other homo- or hetero-dimeric proteins, such as c-Jun/c-Jun, JunD/FosB or JunD/Fra-1 were not. The inhibition of AP-1 activity using morpholino antisense of c-jun mRNAs blocked the *Zic3* expression induced by activin. In addition, co-injection of c-jun mRNA rescued the down-regulated *Zic3* expression. The promoter region of isolated *Zic3* genomic DNA was found to possess several consensus-binding site of AP-1. Thus, in the

functional assays, AP-1 could increase promoter activity of *Zic3* gene. These findings suggest that proneural gene, *Zic3* may be regulated by heterodimeric AP-1(c-Jun/c-Fos) and it may have a role in activin signaling for the regulation of neural specific gene, *Zic3*.

**Keywords:** Activin; AP-1(c-Jun & c-Fos); *Xenopus* development; *Zic3*

### Introduction

The activator protein 1 (AP-1) transcriptional complex is composed of DNA binding proteins belonging to the protooncogenes, Jun and Fos and is the dimer formed between the Jun/Jun or Jun/Fos Family of 'bzip' transcription factors. The Jun family contains three proteins; c-Jun, JunB and JunD while the Fos family consists of five genes; c-Fos, FosB,  $\Delta$ FosB, Fra-1 and Fra-2 (Tulchinsky *et al.*, 2000; Vogt *et al.*, 2001). AP-1 is required for activation of many genes that are induced by treatment of growth factors and cytokines (Shaulian *et al.*, 2001, Kim *et al.*, 2002, Kang *et al.*, 2003). In mesoderm induction, AP-1 was demonstrated as a major downstream mediator in FGF signaling (Dong *et al.*, 1996; Kim *et al.*, 1998) and also in BMP-4 signaling and in BMP-4 expression (Xu *et al.*, 1996; Kim *et al.*, 1998). BMP is a key morphogen functioning in neural inhibition and epidermal differentiation as well as ventral mesoderm specification (Graff *et al.*, 1994; Jones *et al.*, 1996). However, AP-1 involvement has not been reported for the neural specification during the early vertebrate development.

The *Zic* family was originally identified as a group of genes encoding zinc finger proteins in mouse cerebella (Aruga *et al.*, 1994; Aruga *et al.*, 1996; Brown *et al.*, 2003). Also, the *Zic* genes were isolated in human (Herman *et al.*, 2002), chick, (Warner *et al.*, 2003) *Xenopus* (Nakata *et al.*, 1997; Nakata *et al.*, 1998), *Zebrafish* (Grinblat *et al.*, 2001), and *Drosophila* as *odd-paired* (*opa*) gene (Cimborra *et al.*, 1995; Aruga *et al.*, 1996). These zinc finger proteins share a highly conserved domain consisting of five tandemly repeated C<sub>2</sub>H<sub>2</sub>-type zinc finger motifs, which have a role of DNA binding. In *Xenopus*, four *Zic* genes have been isolated and investigated for their properties. Not only in their expression patterns but also in their functions, these *Zic* genes have several

similarities and differences (Nakata *et al.*, 1997; Nakata *et al.*, 1998). Specially, one of these, *Zic3* gene induces neural crest and proneural markers when overexpressed in animal cap explants. In addition, onset of the *Zic3* expression is earlier than most proneural genes and followed chordin expression (Nakata *et al.*, 1997). These data indicated that *Zic3* have a role as a primary regulator both in neural and neural crest development. Recently, *Zic3* was shown to be involved in the left-right specification of the *Xenopus* embryo (Kitaguchi *et al.*, 2000; Herman *et al.*, 2002) and Vg1/activin signaling is involved in this *Zic3*'s effect (Kitaguchi *et al.*, 2000; Weber *et al.*, 2003).

While investigating the roles of AP-1 in *Xenopus* animal cap explant cultures, we found that AP-1 might have a role as a positive regulator of *Zic3* in neural specification. Here, we report the microinjection of a heterodimeric AP-1 (c-Jun/c-Fos) transcription factor but not (c-Jun/c-Jun, JunD/FosB or JunD/Fra-1) leading to the expression of proneural gene, *Zic3*. Further investigations of loss-of-function using morpholino antisenses for c-jun mRNA and of the isolated promoter region *Zic3* genomic DNA, suggest that proneural gene, *Zic3* may be regulated by heterodimeric AP-1(c-Jun/c-Fos) and AP-1 may have a role in activin signaling for the regulation of neural specification.

## Materials and Methods

### Isolation of *Xenopus Zic3* genomic sequence

Genomic clones for *Zic3* were isolated by screening a *Xenopus*  $\gamma$ EMBL4 genomic library (Clontech) with a PCR amplified cDNA probe that corresponds to segment from base pair 91 to 271 of *Xenopus Zic3* cDNA (Genebank AB005292). Approximately  $5 \times 10^5$  clones were screened with a  $^{32}\text{P}$ -labeled probe or ECL system in accordance with the manufacturer's (Amersham) instruction. Thirty putative clones were isolated and analyzed by PCR using the same primers (91-271 nt) used for probe generation. Six clones were selected, which produced a distinct band. We analyzed by Southern hybridization with a *Zic3* cDNA probe, and by digestion with the restriction enzymes. One clone was selected, the fragments generated by *Xho*I and *Hind*III were subcloned into pGEM7 (Promega). The 2.2 kb fragment was further analyzed by complete DNA sequence analysis. Both strands of DNA have been sequenced using automatic sequencing (accession number AF179297).

### Plasmid constructs

Different lengths of the *Zic3* promoters were cloned

into the luciferase reporter plasmid of pGL2-Basic (Promega, Madison, WI). The various *Zic3* promoter constructs were made by cloning 1.8 kb of the promoter into the *Sac*I and blunt-ended *Bgl*III-*Xba*I site of pGL2-Basic and subsequently generating smaller constructs by using specific restriction endonucleases to remove 5' portions of the promoter (Figure 3). -259/ARE *Zic3* promoter construct was made by insertion of activin responsive enhancer region (Weber *et al.*, 2003) behind -259 *Zic3* Luciferase.

### Embryo injection and explant culture

*Xenopus laevis* embryos were obtained by artificial fertilization (Smith *et al.*, 1983). Developmental stages were designated according to Nieuwkoop and Faber (Nieuwkoop *et al.*, 1967). The vitelline membranes were removed by immersing the embryos in the thioglycolic acid solution. Embryos at the one cell stage or two-cell stage were injected in the animal pole with messenger RNA or DNA as described in the figure legends. Animal caps were dissected from the injected embryos at stage 8-9 and cultured to various stages in 67% Leibovitz's L-15 medium (GIBCO/BRL) with L-glutamine (0.3 mg/ml), 7 mM Tris-HCl (pH 7.5) and gentamicin (50 g/ml) for one or two days. Activin were treated in L-15 medium, final concentration 25 ng/ml in the presence of 1 mg/ml bovine serum albumin.

### In vitro transcription

All synthetic mRNAs used for microinjection were produced by *in vitro* transcription. The c-jun cDNA was inserted in the pGEM (Rauscher *et al.*, 1988) and c-fos were sub-cloned into the pSP65 vector (Curran *et al.*, 1987). Each of the cDNAs were linearized and used for *in vitro* synthesis of capped mRNA using *in vitro* transcription kit (Ambion) in accordance with the manufacturer's instructions. The synthetic RNA was quantified by ethidium bromide staining in comparison with a standard RNA.

### RNA isolation and reverse transcription - PCR

Total RNA was extracted from whole embryos or cultured explants with TRIzol reagent (Life Technologies, Inc.) following the manufacturer's instructions. RT-PCR was performed with a Superscript pre-amplification system (Life Technologies, Inc.). PCR was performed as follows: first, a denaturation step of 94°C for 5 min; second, 94°C for 1 min; each annealing temperature, for 1 min; 72°C for 1 min; third, repeat second step 19-28 cycles of amplification was performed as described at the *Xenopus* Molecular Marker Resource (XMMR; University of Texas). All the primer sets of RT-PCR described at the

XMMR were applied.

### Luciferase assay

We have used *Zic3* promoter-Luc in this study. *Zic3* promoter-Luc containing about 1.8 kb genomic DNA was inserted into a luciferase construct with the minimal promoter sequences from albumin gene (Dong *et al.*, 1994; Dong *et al.*, 1995). *Zic3* promoter-Luc plasmid DNA was injected at 20 pg per embryo alone or together with various mRNAs into one-cell-stage embryos or two blastomeres of two-cell-stage embryos. After injection, animal caps were removed at stage 8-9 and cultured in the presence or absence of activin until harvesting at stage 13. Four to five animal caps per group were pooled and homogenized to prepare the cell extract. *Zic3* promoter-dependent luciferase activity of the extract was measured with luminometer at 15 s after mixing of the extract and the Promega luciferase assay reagent. Activity was expressed as integrated light units (Dong *et al.*, 1995).

### Morpholino oligos

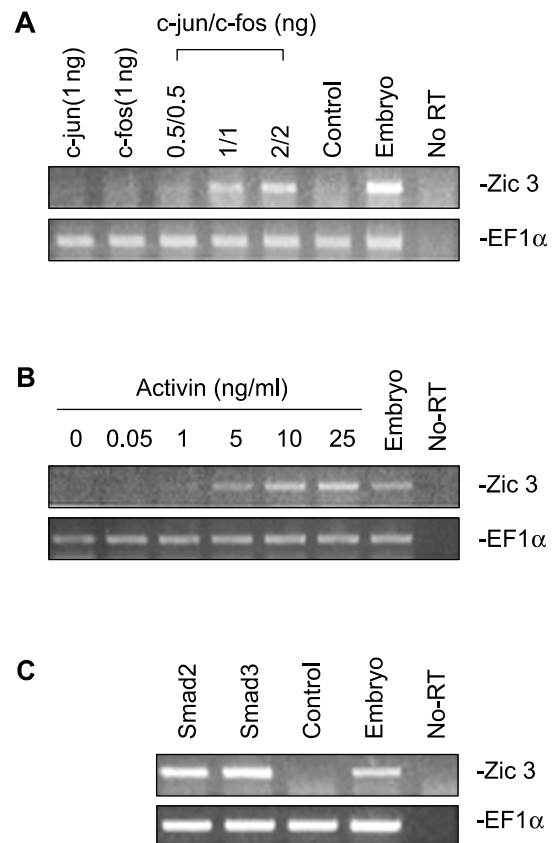
We used morpholino oligos (Gene Tools LLC) for antisense oligodeoxynucleotide. The base composition of antisense oligodeoxynucleotide were 21-mer morpholino 5'CTGGAGCTTATGTCAGTGTGA3' (MO-jun54) and 25-mer morpholino 5'GTAGTTTCCATCTTTGCGTTCATAC3' (MO-jun55). These oligos were designed to bind to complementary sequences found in two kinds of *Xenopus* c-jun mRNAs (Knochel *et al.*, 2000) and the binding prevents translation of those c-jun mRNAs. 5' Morpholino oligos have substitutions of the riboside moieties with nitrogen-containing morpholine moieties and are phosphorodiamidate linked (Summerton *et al.*, 1997). Oligos were resuspended in sterile water and injected in doses of 40 ng per embryos.

## Results and Discussion

### Heterodimeric AP-1(c-Jun/c-Fos) induces *XZic3* in animal cap explants

Previously, it has been demonstrated the involvement of AP-1 in mesoderm induction as well as in neural inhibition during the vertebrate early development (Dong *et al.*, 1996; Kim *et al.*, 1998; Knochel *et al.*, 2000; Peng *et al.*, 2002). However, the roles of AP-1 have not been investigated for the neural induction. In order to examine the roles of AP-1 in neural specification, the expression of proneural gene, *Zic3* expression was investigated. *Zic3* expression was selected because onset of the *Zic3* expression is

earlier than most proneural genes and *Zic3* gene induces neural crest and proneural markers when overexpressed in animal cap explants (Nakata *et al.*, 1997).



**Figure 1.** Heterodimeric c-Jun and c-Fos induces *Zic3* gene in a dose dependent manner. **A:** Co-injection of c-jun and c-fos mRNA induced *Zic3* gene in a dose-dependent manner. *c-Jun* or *c-Fos* alone did not induce the *Zic3* expression. Embryos at one or two cell stages were injected in animal pole regions with mRNAs of the following: lane 1, mRNA of c-jun (1 ng); lane 2, c-fos (1 ng); lane 3, c-jun (0.5 ng) plus c-fos (0.5 ng); lane 4, c-jun (1 ng) plus c-fos (1 ng); lane 5, c-jun (2 ng) plus c-fos (2 ng); lane 6, control  $\beta$ -galactosidase (1 ng). The animal caps were dissected from the injected embryos at stages 8.5-9 and cultured until stage 11. RNAs were isolated from animal cap explants and analyzed by RT-PCR to determine the expression of *Zic3* and the equal loading control *EF1 $\alpha$*  gene using ethidium bromide staining. Embryos at stage 11 were used as a positive control (lane 7), and the same embryo sample processed for RT-PCR in the absence of reverse transcriptase (-rt) was used as a negative control (lane 8). **B:** *Zic3* gene was induced by activin in a dose-dependent manner. The animal caps were dissected from the embryos at stages 8.5-9 and cultured in the absence (lane 1) or in the presence of different dosages of activin (0.05 to 25 ng/ml, lane 2-6) until stage 11. **C:** Smad2 and Smad3, the mediators of activin signaling, also induced the *Zic3* gene. Embryos at one or two cell stages were injected with mRNAs of the following: lane 1, mRNA of Smad2 (1 ng); lane 2, Smad3 (1 ng); lane 3, control  $\beta$ -galactosidase (1 ng). RT-PCR analysis were performed as described in Figure 1A.

Embryos were injected with RNA encoding *c-jun* and/or *c-fos* at one or two cell stages. The animal pole tissue was then dissected at the blastula stage (stage 8-9) and incubated until stage 11. RT-PCR analysis was then performed to examine the expression of *Zic3* as described in Materials and Methods. Injection of  $\beta$ -galactosidase ( $\beta$ -gal) as control RNA, *c-jun*, or *c-fos* RNA alone did not induce any *Zic3* gene expression (Figure 1A). On the other hand animal caps from embryos injected with *c-jun* and *c-fos* RNAs together clearly induced *Zic3* expression (Figure 1A). Moreover, the animal cap explants from the embryos injected with other heterodimeric AP-1 components of JunD/Fra-1 or JunD/FosB RNA combinations did not induce *Zic3* gene (data not shown). The results clearly demonstrated that heterodimeric AP-1 (*c-Jun* and *c-Fos*) is sufficient to induce *Zic3* gene in animal cap explants.

Previously, the involvement of AP-1 in mesoderm induction was demonstrated as a major downstream mediator of FGF signaling (Dong *et al.*, 1996; Kim *et al.*, 1998). However, FGF signaling alone in animal cap explants does not induce *Zic3* gene expression. Therefore, *Zic3* expression by heterodimeric AP-1 (*c-Jun* and *c-Fos*) was somewhat unexpected result. *Zic3* expression in animal cap explants is similar to activin activity, but different from FGF activity.

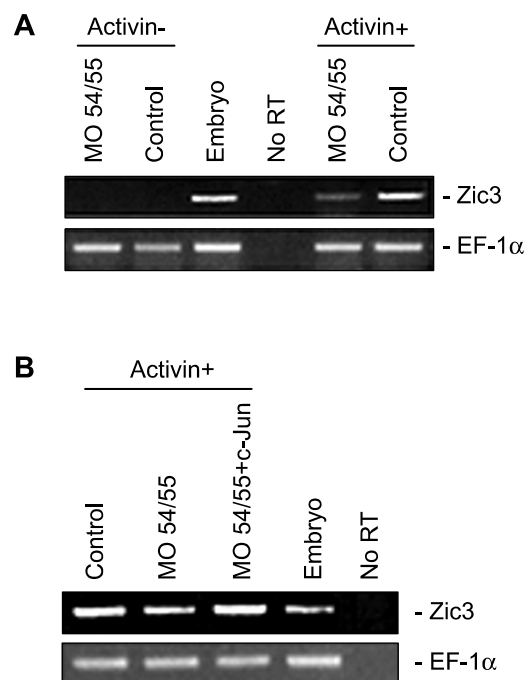
We confirmed *Zic3* induction by activin (Figure 1B) and the signal mediators of activin signaling (Smad2 and Smad3) (Figure 1C). Embryos were injected with  $\beta$ -gal or RNA encoding Smad2 or Smad3. The animal pole tissues were dissected at the blastula stage (stage 8-9) and incubated until stage 11. The animal cap tissues from embryos injected with  $\beta$ -gal RNA were incubated in the absence or in the presence of different dosages of activin protein. *Zic3* gene expression was induced in activin treated animal cap explants in a dose dependent manner (Figure 1B) as well as in the animal cap explants obtained from the embryos injected with RNA encoding Smad2 or Smad3 (Figure 1C). Taken together, the results suggest that heterodimeric AP-1 (*c-Jun* and *c-Fos*) may be involved in neural specification of *Zic3* induction in activin signaling.

### The blocking of AP-1 decreases the expression of *Zic3* gene induced by activin

Co-expression of *c-Jun* and *c-Fos* induced *Zic3* gene expression and the effect was similar to the activity of activin signaling in animal cap explants, but different from that of FGF signaling. In order to examine the *Zic3* regulation by heterodimeric AP-1 (*c-Jun/c-Fos*) in activin signaling, the effect on the AP-1 function was examined in the animal cap explant culture system of *Xenopus* embryos.

For effective blocking of AP-1 activity in *Xenopus* embryos, we used morpholino oligos of *c-jun* mRNAs (MO-*c-Jun*54 and MO-*c-Jun*55) as described in Materials and Methods. Morpholino oligos have a function of antisense by preventing translation of mRNA. For the reasons of non-toxicity, stability and permeability, advanced antisense structural type of morpholino oligos recently have been used (Gene Tools LLC). Here, we used MO-*c-Jun*54 and MO-*c-Jun*55. These morpholinos were designed to bind to 5' untranslated regions including the AUG translational start sites in two kinds of *Xenopus c-jun* mRNAs (*c-Jun*54 and *c-Jun*55). We injected 40 ng of MO-*c-Jun*54 and MO-*c-Jun*55 (MO54/55) into one or two cell stage, then dissected animal caps at stage 8-9 and cultured them in the presence or absence of activin. Then we analyzed the *Zic3* expression using RT-PCR.

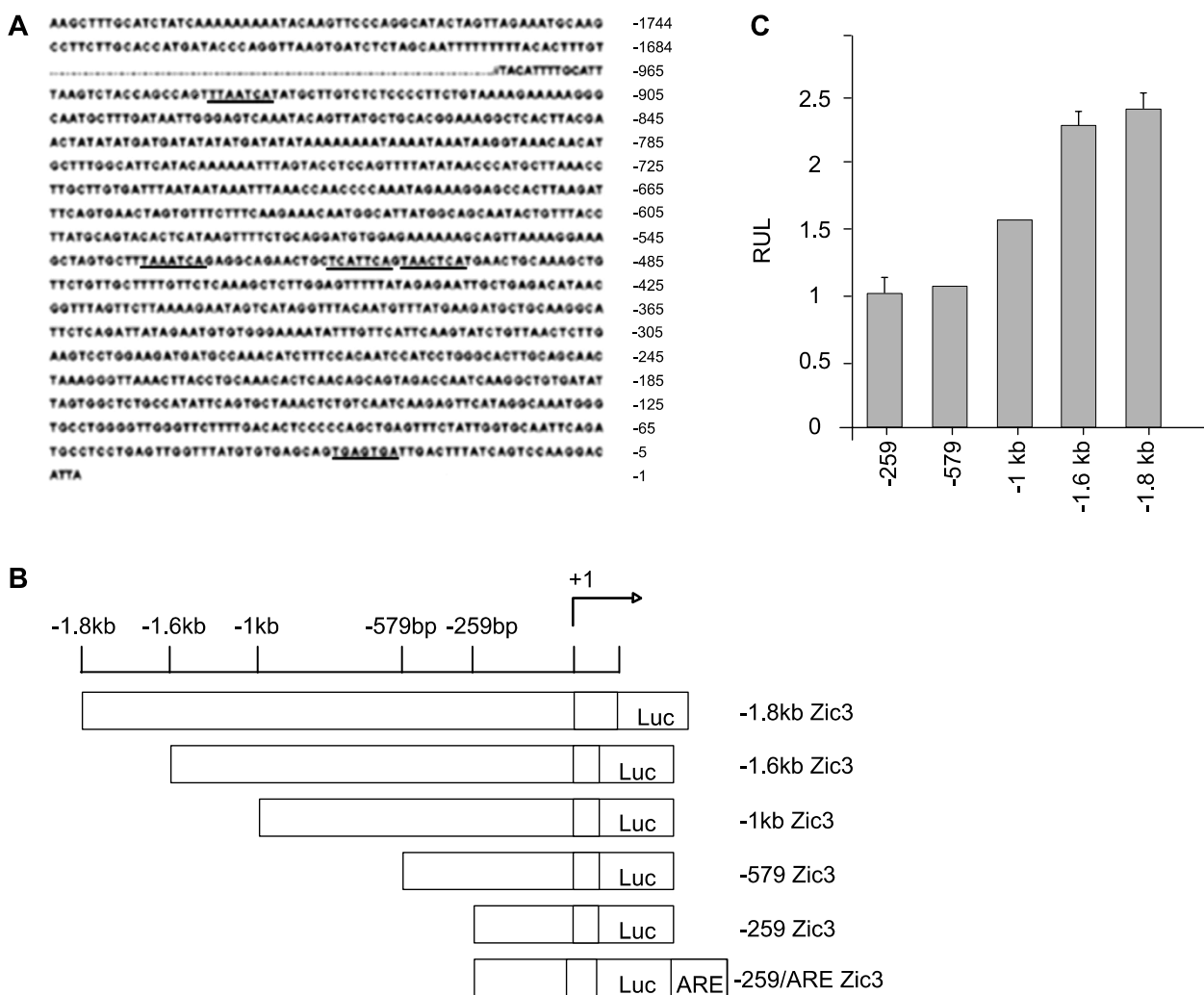
As expected, the blocking of AP-1 activity by MO-*c-Jun*s resulted in down-regulation of *Zic3* gene induced by activin (Figure 2A). In order to confirm that



**Figure 2.** The blocking of AP-1 decreases the expression of *Zic3* gene induced by activin. (A) Blocking of AP-1 activity decrease *Zic3* gene induced by activin. Animal pole tissues injected with morpholino *c-Jun*s [MO-54 (40 ng) and MO-55 (40 ng) *c-Jun*s, per embryos] were cultured in the presence or absence of activin (25 ng/ml). *Zic3* gene induction was examined at stage 11 by RT-PCR as described in Figure 1A. MO-*c-Jun*s inhibited *Zic3* expression induced by activin. (B) The *Zic3* expression inhibited by MO-*c-Jun*s was rescued by co-injection of rat *c-jun* mRNA. Animal pole tissue injected with morpholino *c-Jun*s alone or co-injection of *c-jun* mRNA (1 ng) were cultured in the presence of activin (25 ng/ml). *Zic3* gene expression was examined at stage 11 by RT-PCR as described in Figure 1A.

the blocking activities of MO-c-Juns against endogenous c-jun mRNAs were specific, we tested whether co-injection of rat c-jun mRNA could rescue the Zic3 expression decreased by MO-c-Juns. The overexpression of rat c-jun indeed rescue the Zic3 expression decreased by MO-c-Juns, suggesting that MO-c-Juns blocked against the endogenous c-jun mRNAs in specific mood (Figure 2B). Two kinds of c-jun mRNAs have been reported in Xenopus em-

bryos (Knochel et al., 2000). Interestingly, we found that one form of c-Jun Morpholino alone was not effective for blocking of c-Jun activity, indicating that both of mRNAs may be functioning during the early neural specification caused by activin signaling in Xenopus embryos. Taken together, the results suggested that heterodimeric AP-1 (c-Jun and c-Fos) is involved in Zic3 expression induced by activin signaling.



**Figure 3.** The isolated 5' flanking region of *Xenopus Zic3* gene. (A) DNA sequence of *Xenopus Zic3* 5' flanking region (GeneBank Accession No. AF179297). Sequence motifs for the putative AP-1 binding sites are indicated as underlined bold letters. The numbers refer to the position relative to the transcriptional start site (+1). (B) A schematic diagram illustrating the *Zic3* promoter-reporter deletion constructs. Serial deletion constructs of the *Zic3* promoter were subcloned into pGL2-basic plasmid. The transcription start site is indicated at +1 and Luc box represents the luciferase gene. ARE indicates activin responsive enhancer region which was published by Weber et al., 2003. (C) Relative luciferase activity of 5' serial deletion constructs of genomic *Zic3*. Basal activity in animal cap was a little decreased as promoter shortens. *Zic3* promoter-Luc plasmid DNAs were injected at 20 pg per embryo into one-cell-stage embryos or two blastomeres of two-cell-stage embryos. After injection, animal caps were removed at stage 8-9 and cultured until harvesting at stage 13. Four to five animal caps per group were pooled and homogenized to prepare the cell extract. The relative luciferase activity of the extract was measured with luminometer at 15 s after mixing of the extract and the Promega luciferase assay reagent. The relative activity was expressed as integrated light units.

### Isolation of 5' flanking region of *Xenopus Zic3* gene

*Zic3* gene was induced by heterodimeric AP-1 (c-Jun and c-Fos) and *Zic3* expression by activin signaling was blocked by MO-c-Juns. In order to examine the transcriptional regulation of *Zic3* gene by heterodimeric AP-1 (c-Jun and c-Fos) in the isolated genomic DNA, we isolated genomic *Zic3* gene from the *Xenopus* muscle genomic library.

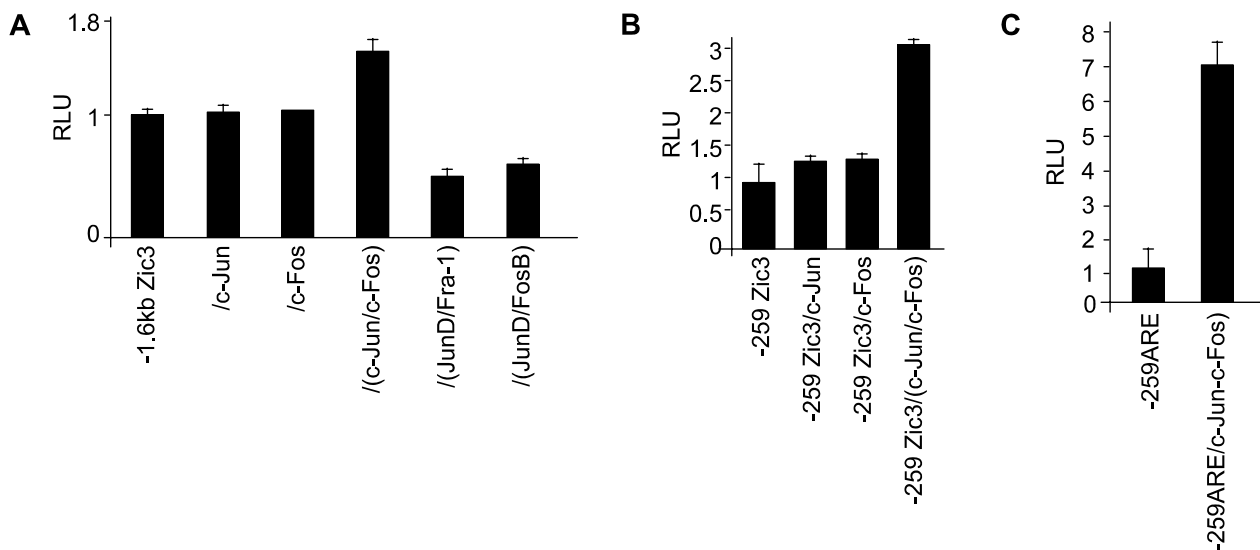
The *Xenopus* muscle genomic library was screened with the *Zic3* 5' UTR specific probe (see details in Materials and Methods). A 2041 bp genomic fragment containing *Zic3* 5' UTR region was subcloned and sequenced. Figure 3A shows a schematic diagram of the genomic organization of *Xenopus Zic3* gene. This genomic sequence covered 100 bp of coding region, 130 bp of UTR region and about 1.8 kb of 5' flanking region (Gene bank accession number AF179297). Many putative transcriptional regulatory elements were identified within the 5' flanking region of *Zic3*. Interestingly, we identified five putative AP-1 binding sites (Figure 3A). The presence of putative binding site for the AP-1 supports that AP-1 transcriptional factor may be involved in the regulation of *Zic3* gene.

In order to further study of transcriptional regulation of *Zic3*, we constructed the series of *Zic3* 5' deletion constructs reaching to position -259 relative to the transcription initiation site (Figure 3B) and tested basal activity of these constructs (Figure 3C). The basal activities of -1.8 kb and -1.6 kb *Zic3* constructs were high and similar. The basal activities of other deletion constructs were decreased (Figure 3C).

### Activation of *Zic3* promoter activity by AP-1 (c-Jun/c-Fos)

*Zic3* gene was induced by heterodimeric AP-1 (c-Jun and c-Fos). In order to examine whether the isolated *Zic3* promoter was also regulated by heterodimeric AP-1 (c-Jun and c-Fos), the *Zic3* promoter-Luc reporter genes were co-injected with the mRNAs encoding c-Jun and c-Fos or injected with other AP-1 components at one or two cell stage. The animal pole tissue was then dissected at stage 8-9 and incubated until stage 13 to measure the reporter activities.

As expected, the relative reporter activity of -1.6 kb of *Zic3* promoter was enhanced by co-injection of *c-jun* and *c-fos* mRNAs together. On the other hand,



**Figure 4.** Heterodimeric c-Jun and c-Fos increases *Zic3* promoter activity. (A) The embryos were co-injected at one cell stage into the animal pole region with 20 pg per embryo and indicated mRNAs (1 ng per embryo) with *Zic3* promoter-Luc reporter construct of -1.6 kb *Zic3* promoter. Animal caps were dissected from the embryos at stage 8.5 and cultured until stage 11. The injected mRNAs included mRNAs of *c-jun*, *c-fos*, *jun D*, *fra-1* and *fosB*. Animal pole tissues were cultured until stage13 and luciferase activities were measured as described in Figure 3. The reporter activity of -1.6 kb *Zic3* promoter construct was activated only by co-injection of *c-jun* and *c-fos* RNA together. However *c-jun* or *c-fos* alone did not activate the reporter activity of -1.6 kb *Zic3* promoter construct. Moreover co-injection of other AP-1 components of Jun D/Fra-1 or JunD/FosB reduced the reporter activity of -1.6 kb construct. (B) The embryos were co-injected at one cell stage into the animal pole region with 20 pg of -259-Luc reporter gene and indicated mRNAs (1 ng per embryo). Animal pole tissues were cultured until stage13 and luciferase activities were measured as described in Figure 3. The reporter activity of -259-Luc reporter construct was increased by co-injection of *c-jun* and *c-fos*, but not by *c-jun* or *c-fos* alone. (C) The embryos were co-injected at one cell stage into the animal pole region with 20 pg of -259ARE-Luc reporter gene and indicated mRNAs (1 ng per embryo). Animal pole tissues were cultured until stage13 and luciferase activities were measured as described in Figure 3. The shortest *Zic3* promoter, -259-Luc reporter gene containing ARE was also increased by co-injection of *c-jun* and *c-fos*.

animal caps from embryos injected with  $\beta$ -galactosidase ( $\beta$ -gal) as control RNA, *c-jun* or *c-fos* RNA alone did not increase the reporter activity (Figure 4A). Moreover, the animal cap explants from the embryos injected with other heterodimeric AP-1 components of *junD/fra-1* or *junD/fosB* RNA combinations did not increase the reporter activity. In addition, the reporter activity of shortest 5' deletion construct of -259 *Zic3* promoter-Luc was also enhanced only by co-injection of *c-jun* and *c-fos* (Figure 4B). Although whether the enhancement of the reporter gene activity by heterodimeric AP-1 is direct or indirect, is not conclusive, the results were consistent with *Zic3* induction experiment as shown in Figure 1C, suggesting that the isolated *Zic3* promoter may contain the region regulated by AP-1.

A significant level of activity enhancement by heterodimeric AP-1 (*c-Jun/c-Fos*) remained even in the -259 Luc construct. Recently, an activin responsive enhancer (ARE) region, which was located in the *Zic3* first intron, was identified as one of *Zic3* gene regulatory element (Weber *et al.*, 2003). In order to examine the effect of ARE region for heterodimeric AP-1, we constructed -259 Luc-containing ARE region (-259ARE) (Figure 3B). Interestingly, the reporter activity enhancement containing ARE in -259 *Zic3* Luc construct was greater than that of -259 *Zic3* Luc construct by heterodimeric AP-1 (*c-Jun/c-Fos*), supporting the results of AP-1 involvement in *Zic3* expression by activin signaling shown in Figure 2.

In this paper, we show, for the first time, that heterodimeric AP-1 (*c-Jun/c-Fos*) induces the expression of *Zic3* gene, a primary proneural gene in animal cap explants. Since *Zic3* gene has significant roles both in neural and neural crest development in *Xenopus* embryo, the results suggest that heterodimeric AP-1 (*c-Jun/c-Fos*) may have a role in neural specification during the early vertebrate development. In addition, heterodimeric AP-1 (*c-Jun* and *c-Fos*) is involved in *Zic3* expression induced by activin signaling by using specific antisense Morpholino *c-Juns*. The involvement of heterodimeric AP-1 in *Zic3* expression by activin was somewhat unexpected results since heterodimeric AP-1 has been reported to be an essential component of FGF signaling, however, AP-1/*Jun* has been reported not to mediate mesoderm induction by activin. AP-1 involvement in mesoderm and neural specification may be different. Although the detail studies of AP-1 involvement in activin activity is beyond the scope of this paper, it will be interesting to further investigate the role of AP-1 in activin signaling during the early development of *Xenopus* embryos. By screening of 1.8 kb 5' flanking region of *Zic3* gene and the reporter activity shown to be enhanced by heterodimeric AP-1 (*c-Jun/c-Fos*), provided a strong supports for the AP-1 involvement

in *Zic3* expression. Other heterodimeric AP-1 components of AP-1 did not increase the activity of *Zic3* promoter nor *Zic3* expression. The results suggest that *Zic3* regulation by AP-1 is dependent on heterodimeric AP-1 consisted of *c-Jun* and *c-Fos*, not on other AP-1 components (*JunD/FosB* and *JunD/Fra-1*). Various combinations of different AP-1 components were found to have distinctive roles during the early *Xenopus* development (unpublished data). Taken together, these results demonstrate that heterodimeric AP-1 (*c-Jun/c-Fos*) is a key molecule in *Zic3* expression in the neural specification during the early development of vertebrate embryos.

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