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AP-1$^{c\text{-}Jun/FosB}$ mediates xFoxD5b expression in *Xenopus* early developmental neurogenesis

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ABSTRACT AP-1 (activator protein-1) is composed of Jun and Fos proteins and functions in cell proliferation, apoptosis and differentiation. Previous studies have demonstrated that different AP-1 complexes participate in the determination of various cell fates. However, the role of different AP-1 complexes during early vertebrate development is not yet fully understood. In the present study, we demonstrate that AP-1$^{c\text{-}Jun/FosB}$ regulates neurogenesis via FoxD5b expression. We show that FoxD5 was induced by the inhibition of BMP and that FoxD5b plays roles in neurogenesis. Additionally, we show that the FoxD5b promoter region within -1336 and -1316 contains an AP-1 binding site, which is required for the transcriptional regulation of FoxD5b and is induced by the inhibition of BMP signaling in animal cap explants. Moreover, c-Jun, a component of AP-1, directly binds to the AP-1 binding site of the FoxD5b promoter and induces FoxD5b expression cooperatively with FosB, but not with c-Fos or Fra-1. Altogether, these results suggest that AP-1$^{c\text{-}Jun/FosB}$ may play a role in neurogenesis via the induction of FoxD5b expression during early vertebrate development.

KEY WORDS: *AP-1$^{c\text{-}Jun/FosB}$, FoxD5b, BMP, Neurogenesis, Xenopus*

Introduction

AP-1 (activator protein-1) is a transcription factor that is involved in proliferation, transformation and apoptosis in response to various stimuli (Angel and Karin, 1991, Hess *et al*., 2004, Shaulian and Karin, 2001). AP-1 is a heterodimeric complex that is composed of Jun and Fos family proteins; it is known that Fos proteins can only heterodimerize with Jun protein, but that Jun can homodimerize with itself and heterodimerize with Fos protein (Glover and Harrison, 1995, Smeal *et al*., 1989). AP-1 induces its target genes via binding consensus sequences (5`-TGAG/CTCA-3`) through a leucine zipper domain (Rauscher *et al*., 1988). Although numerous studies have demonstrated the function of AP-1, the diverse roles of AP-1 in differentiation and development are largely unknown. Recent studies have suggested that AP-1s may have opposite and overlapping functions in proliferation and apoptosis (Ameyar *et al*., 2003). In early *Xenopus* development, AP-1$^{cJun/c\text{-}Fos}$ induces the expression of organizer and neural genes on the dorsal side of the embryo, while AP-1$^{JunD/c\text{-}Fos}$ regulates hematopoiesis through BMP signaling on the ventral side (Lee *et al*., 2004, Lee *et al*., 2011, Lee *et al*., 2012).

During vertebrate embryonic neurogenesis, BMP acts as a negative regulator of neurogenesis and as an epidermal inducer (Dale and Jones, 1999, Dale and Wardle, 1999, Dosch *et al*., 1997, Glinka *et al*., 1997). The ectopic expression of dominant-negative BMP receptor (DNBR) increases the expression of various neural genes without affecting the expression of mesodermal and organizer genes (Xu *et al*., 1995). The function and regulation of genes induced by BMP have been well studied in the formation of the ventral mesoderm and epidermis. However, gene profiles induced by the inhibition of BMP signaling need additional works. Moreover, how target genes in the neural induction process are affected by the inhibition of BMP signaling remain unclear. In the present study, we report that FoxD5b is a candidate neural factor induced by the inhibition of BMP during *Xenopus* embryogenesis.

FoxD5b is a member of the Fox subfamily (http://biology.pomona.edu/fox/). Fox genes have a conserved winged helix (or forkhead)

Abbreviations used in this paper: AP-1, activator protein-1, BMP, bone morphogenetic protein; DNBR, dominant-negative BMP receptor.

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domain and play important roles in various cellular processes (Jackson *et al.*, 2010, Katoh *et al.*, 2012, Katoh and Katoh, 2004, Pohl and Knochel, 2005). FoxD5, a well-known neural gene, acts as a transcription factor in the nucleus (Solter *et al.*, 1999). The FoxD5 gene is involved in various biological processes such as transformation, metabolism and development (Katoh and Katoh, 2004, Lee *et al.*, 2009). At the first, the FoxD5 was isolated and identified as XFLIP (*Xenopus* forkhead expressed in the dorsal lip) which is related to the *Drosophila* FKH sequence (King and Moore, 1994). Later, the XFLIP has been classified into sub-families, termed XFD-12, XFD-12' and XFD-12" (Solter *et al.*, 1999). It has been shown that FoxD5a is involved in the initiation of neural induction, but not in neuronal differentiation (Neilson *et al.*, 2012, Yan *et al.*, 2009a, Yan *et al.*, 2009b, Yu *et al.*, 2002). Recently, Neilson *et al.,* demonstrated that FoxD5 regulates the transition of neuroectoderm via controlling a network of genes (Neilson *et al.*, 2012). Furthermore, canonical Wnt, activin and FGF signaling transcriptionally regulate FoxD5a (Solter *et al.*, 1999, Sullivan *et al.*, 2001). However, to date, the function and transcriptional regulation of FoxD5b during early *Xenopus* development have not yet been elucidated. Here, we report that FoxD5b is induced by the inhibition of BMP and plays a role in neurogenesis during *Xenopus* embryogenesis. We isolated the 5'-flanking region (-1336 bp) of FoxD5b to analyze the regulatory element in the FoxD5b promoter, which is activated when BMP signaling is inhibited during early *Xenopus* development. We identified a positive cis-acting element in the FoxD5b promoter that responded to AP-1$^{c\text{-}Jun/FosB}$ and showed that this site played a critical role in enhancing the FoxD5b expression that is caused by the inhibition of BMP. Altogether, these results suggest that AP-1$^{c\text{-}Jun/FosB}$ plays an essential role in the induction of xFoxD5b caused by the inhibition of BMP signaling during *Xenopus* embryonic neurogenesis.

Results

FoxD5b is expressed by dominant-negative BMP receptor (DNBR) and induces neurogenesis in the ectodermal explants of Xenopus *embryos*

We performed Affymetrix Gene Chip analysis in animal cap explants to identify genes that are induced during early neurogenesis due to the inhibition of BMP signaling(Hocking and McFarlane, 2007, Wilson and Hemmati-Brivanlou, 1995). As shown in Table 4, the microarray data indicated that the ectopic expression of DNBR induced several neural genes including Zic3, xCYP26c, Sox2/3 and FoxD5a/b (Cunningham *et al.*, 2008, El-Hodiri *et al.*, 2001, Sasai, 2001, Tanibe *et al.*, 2008). We confirmed the quality of the sample used for the microarray. The ectopic expression of DNBR induced Zic3, an early neural gene, without inducing Xbra (pan-mesoderm) or Chordin (organizer gene) in animal cap explants (Fig. 1A). Additionally, the direct target of BMP signaling, xVent-1, was repressed by DNBR. Consistent with the microarray data, the ectopic expression of DNBR significantly induced both FoxD5a and FoxD5b. Furthermore, we also showed that ectopic expression of DNBR increased the FoxD5b expression in whole embryo using *in situ* hybridization assay (Fig. 1B). Previous studies reported that FoxD5a played a role in maintaining undifferentiated neural ectoderm after neural induction (Yan *et al.*, 2009a). However, the function of FoxD5b, a putative pseudo-allele gene of FoxD5a, has not yet been studied. Therefore, we performed a gain-of-function study of FoxD5b. The overexpression of FoxD5b in animal cap explants significantly induced SOX2 expression at stage 10.5 and NCAM and NeuroD and N-tubulin dose dependently at stage 24; however, OTX2 was not induced (Fig. 1B). Although FoxD5a suppresses neural differentiation genes in whole embryos, the overexpression of FoxD5a induces several neural differentiation genes in animal cap explants (Sullivan *et al.*, 2001). Similar to FoxD5a, FoxD5b also induced Sox2, NCAM, NeuroD and N-tubulin in animal cap explants. Altogether, these data suggest that FoxD5b is induced by the inhibition of BMP signaling and promotes neurogenesis.

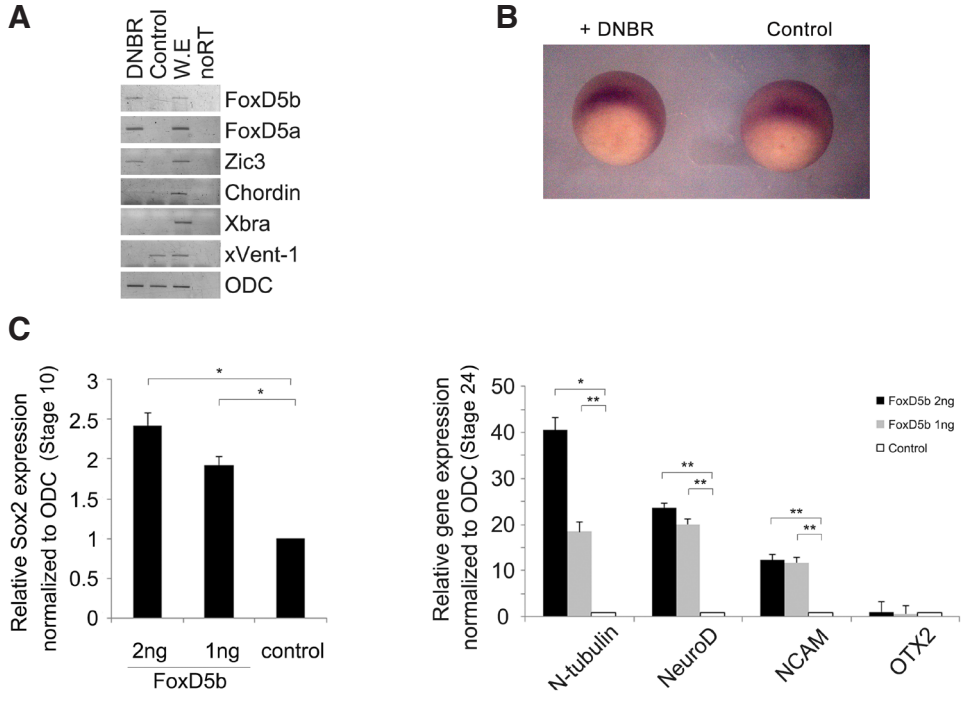

Fig. 1. FoxD5b is induced by dominant-negative BMP receptor (DNBR) and promotes neurogenesis in animal cap explants. (A) *DNBR RNA (1ng) was injected into the animal pole region at the one-cell stage. Animal caps were dissected at stage 8 and incubated until stage 10. RT-PCR was performed for the analysis of the expression of the indicated genes.* **(B)** *Embryos injected with DNBR mRNAs (1ng) were processed for whole mount* in situ *hybridization with anti-sense probe of FoxD5b at stage 11.* **(C)** *FoxD5b RNA (2 or 1 ng) was injected into the animal pole region at the one-cell stage. The animal caps were dissected at stage 8 and incubated until stage 10 or 24. qRT-PCR was performed for the analysis of the expression the indicated genes. *,* p *value < 0.05, **,* p *value < 0.01.*

The transcriptional activity of FoxD5b is increased by the inhibition of BMP signaling

Previously, the 5' and 3' flanking regions of FoxD5 were isolated, and the elements that contribute their temporal and spatial expression were analyzed

(Schon *et al*., 2004). This study showed that the 3'-flanking region possesses enhancer activity and cooperates with the proximal upstream region. However, they did not identify any cis- or trans-acting elements for the 5'-flanking region of FoxD5. To investigate the transcriptional regulation of the 5'-flanking region of FoxD5b in detail, we isolated the -1336/+96 fragment of the FoxD5b gene. We then investigated whether the 5'-flanking region of FoxD5b is enhanced by the inhibition of BMP. The promoter activity of -1336 bp construct was measured in the absence or presence of DNBR mRNA. Consistent with the RT-PCR data, the promoter activity of -1336 bp construct was enhanced approximately 4 fold by co-injection of DNBR mRNA (Fig. 2B). To identify the response element for DNBR in the 5'-flanking region of FoxD5b, we produced various truncated or point-mutated constructs as described in the Materials and Methods. We performed a luciferase assay using a serial deletion of the 5'-flanking region (Fig. 2A) and compared the promoter activities of our serial-truncated mutants when BMP was inhibited. The reporter activity of -1336 construct was increased by DNBR, but the positive response to DNBR was completely abolished in the -1316 construct (Fig. 2C). These results indicate that the region between -1336 and -1316 is critical for the positive transcriptional regulation of FoxD5b by DNBR.

The AP-1 binding site is important for the DNBR-induced FoxD5b expression

To identify the regulatory element between -1336 and -1316, we searched for a putative transcription factor binding site. Interestingly, a putative AP-1 binding site (TGACTC) and a Vent-1 binding site (CAAATAA) were located in this region (Kim *et al*., 2006, Taylor *et al*., 2006). AP-1 is a transcriptional activator, and Vent-1 is a repressor and functions as a BMP target gene. To evaluate which site is critical for the transcriptional regulation of FoxD5b that is enhanced by the inhibition of BMP signaling, we generated two -1336 constructs that contained mutated sequences in the putative Vent-1 binding site (1336-mVent-1) and AP-1 binding site (1336-mAP-1) using site-directed mutagenesis (Fig. 2A). We then measured the promoter activities of wild-type -1336, -1336-mAP-1 and -1336-mVent-1 in animal caps derived from embryos injected with each construct alone or co-injected with DNBR. As shown in Fig. 2D, the -1336-mAP-1 construct completely lost the ability to positively respond to DNBR. However, when compared with the luciferase activity of -1336, -1336-mVent-1 luciferase activity was the same in the presence and absence of DNBR (Fig. 2D). This result indicated that the AP-1 binding site plays a role as a positive-cis-acting element in the 5'-flanking region of FoxD5b. In addition, we examined whether c-Jun protein directly binds to the putative AP-1 binding site of the 5'-flanking region of FoxD5b through a ChIP assay performed with anti-c-Jun antibody. As shown in Fig. 2E, c-Jun directly binds to this region. Altogether, these data suggest that the AP-1 binding site within -1336 and -1316 of the FoxD5b promoter is required for the DNBR-induced transcriptional regulation of FoxD5b.

The AP-1 component, c-Jun, is required for DNBR-induced FoxD5b expression

Next, to confirm that c-Jun is required for DNBR-induced FoxD5b expression in animal cap explants, we examined the reporter activ-

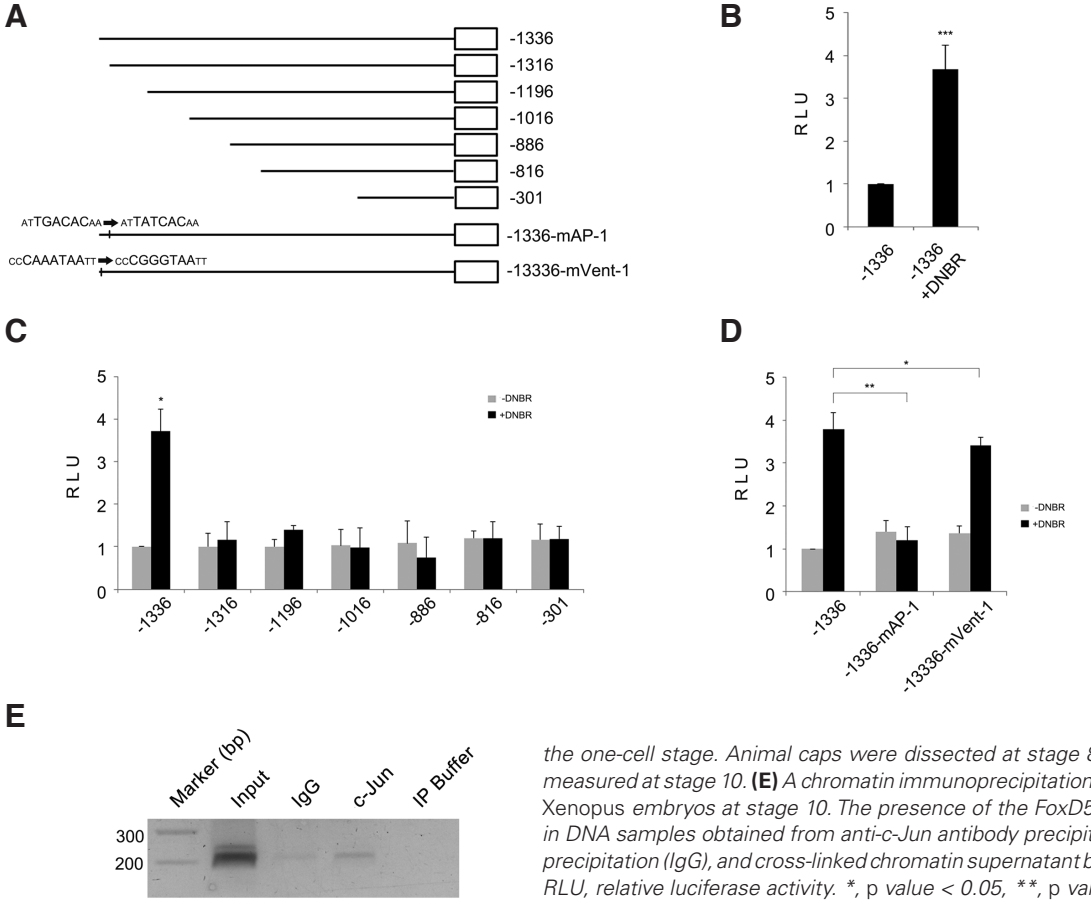

Fig. 2. The AP-1 binding site has a critical role in dominant-negative BMP receptor (DNBR)-induced FoxD5b expression. (A) *Schematic representation of FoxD5b serially truncated constructs and a point mutation of AP-1 and Vent-1 putative binding site constructs* **(B)**. *Embryos were co-injected with the -1336 construct (20pg) and DNBR (1ng) at the one-cell stage and incubated until stage 10. Luciferase activity was measured as described in Methods.* **(C)** *Various truncated constructs (20pg) were either injected alone or co-injected with DNBR (1ng) at the one-cell stage. Animal cap explants were dissected at stage 8, incubated until stage 10 and then luciferase activity was measured.* **(D)** *The -1336, AP-1 or Vent-1 site mutants were either injected alone or co-injected with DNBR (1ng) at the one-cell stage. Animal caps were dissected at stage 8, and their luciferase activity was measured at stage 10.* **(E)** *A chromatin immunoprecipitation (ChIP) assay was performed using* Xenopus *embryos at stage 10. The presence of the FoxD5b promoter was detected by PCR in DNA samples obtained from anti-c-Jun antibody precipitation (c-Jun), normal IgG antibody precipitation (IgG), and cross-linked chromatin supernatant before immunoprecipitation (Input). RLU, relative luciferase activity. *,* p *value < 0.05, **,* p *value < 0.01; ***,* p *value < 0.001.*

ity in the presence of c-Jun anti-sense morpholino-oligos (54/55). The co-injection of 54/55 morpholinos significantly reduced the DNBR-enhanced luciferase activity of -1336, while the control scrambled morpholino-oligos (SC) did not (Fig. 3A). In addition, we confirmed the reporter data with actual FoxD5b expression using qRT-PCR. The co-injection of 54/55 with DNBR reduced FoxD5b expression in animal cap explants, but co-injection of SC with DNBR did not (Fig. 3B). Interestingly, the co-injection of 54/55 with DNBR significantly reduced the expression of NCAM, NeuroD and N-tubulin, but not OTX2 (Fig. 3C). These results were consistent with the data shown in Fig. 1B—the overexpression of FoxD5b increased the expression of NCAM, NeuroD and N-tubulin, but not OTX2. The knockdown of c-Jun reduced FoxD5b expression and selectively led to a decrease of NCAM, NeuroD and N-tubulin expression. Altogether, our data demonstrate that AP-1 plays an important role in DNBR-enhanced FoxD5b expression.

c-Jun and FosB cooperatively induce FoxD5b expression

Our previous studies have demonstrated that the ectopic expression of c-Jun alone did not induce organizer or neural genes (Lee *et al.*, 2004, Lee *et al.*, 2011). Thus, we expected that c-Jun and a Fos family protein may cooperatively induce FoxD5b expression. To examine which Fos protein cooperatively participates with c-Jun to induce FoxD5b expression, we co-injected c-Jun with different Fos family members including c-Fos, FosB and Fra1. As shown in Fig. 4A, the co-injection of c-Jun with FosB significantly increased FoxD5b expression. In addition, the enhanced expression of Fox5b observed in the qRT-PCR data with FosB was confirmed by the separated FoxD5b promoter assay with -1336 (Fig. 4B). Interestingly, the co-injection of AP-1$^{c\text{-}Jun/FosB}$ with DNBR synergistically induced FoxD5b expression and the luciferase activity of -1336 (Fig. 4C and D). We further examined the relationship between AP-1$^{c\text{-}Jun/fosB}$ and the inhibition of BMP signaling in FoxD5b expression during neurogenesis. First, we evaluated the level of AP-1transcription; however, the overexpression of DNBR did not increase the expression level of c-Jun or FosB (data not shown). Thus, we measured whether DNBR increased the activity of AP-1$^{c\text{-}Jun/FosB}$ with AP-1 Luc, which contains AP-1 response elements. As shown in Fig. 4E, when compared to the control, DNBR alone increased AP-1 activity approximately 2 fold, and the ectopic expression of AP-1 increased the activity approximately 10 fold. Interestingly, the co-injection of DNBR and AP-1$^{c\text{-}Jun/fosB}$ synergistically increased AP-1 activity. We then observed that the phosphorylation at Ser63 of c-Jun was increased by ectopic expression of DNBR (Fig. 4F). This result suggests that suppression of BMP signaling increases AP-1$^{c\text{-}Jun/FosB}$ activity. Next, we examined whether the AP-1$^{c\text{-}Jun/FosB}$ also induced FoxD5b expression in whole embryo. *In situ* hybridization data showed that over-expression of AP-1$^{c\text{-}Jun/FosB}$ increased the FoxD5b expression (Fig. 4G). We showed that c-Jun protein directly binds to the 5'-flanking region of FoxD5b (Fig. 2E). To verify that FosB also binds to this region, we performed ChIP assay with flag tagged FosB and Flag specific antibodies. As shown in Fig. 4H, FosB bound to the 5'-flanking region of FoxD5b.

Overall, our data suggest that the heterodimer

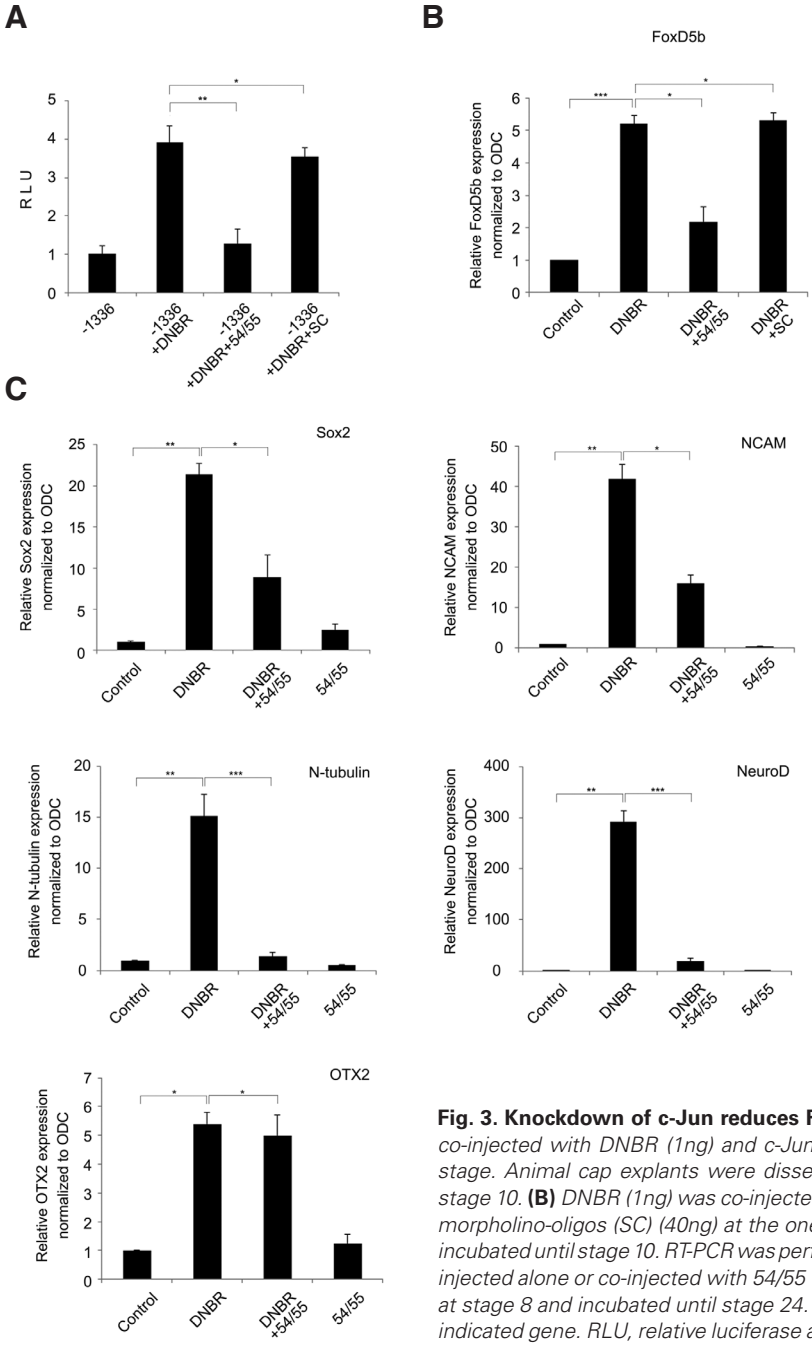

Fig. 3. Knockdown of c-Jun reduces FoxD5b and neural gene expression. (A) *Wild-type -1336 was co-injected with DNBR (1ng) and c-Jun morpholino-oligos (54/55) (40ng), as indicated, at the one-cell stage. Animal cap explants were dissected at stage 8, and their luciferase activity was measured at stage 10.* **(B)** *DNBR (1ng) was co-injected with c-Jun morpholino-oligos (54/55) (40ng) or standard control morpholino-oligos (SC) (40ng) at the one-cell stage. Animal cap explants were dissected at stage 8 and incubated until stage 10. RT-PCR was performed for the analysis of FoxD5b expression.* **(C)** *DNBR (1ng) was injected alone or co-injected with 54/55 (40ng) at the one-cell stage. Animal cap explants were dissected at stage 8 and incubated until stage 24. RT-PCR was performed for the analysis of the expression of the indicated gene. RLU, relative luciferase activity. *,* p *value < 0.05, **,* p *value < 0.01; ***,* p *value < 0.001.*

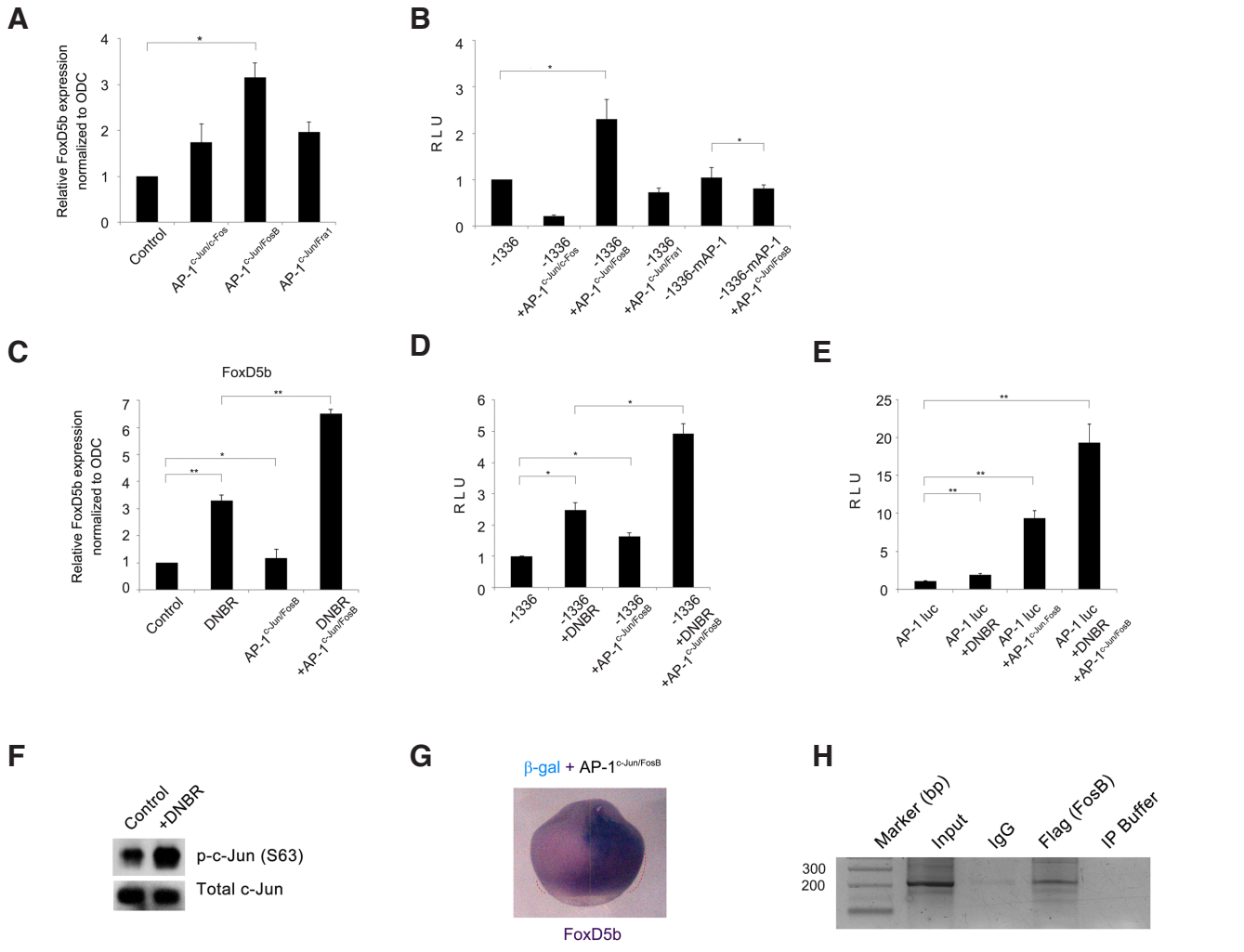

Fig. 4. An AP-1 complex composed of c-Jun and FosB induced FoxD5b expression. (A) *AP-1 RNA (1ng), as indicated, was injected at the one-cell stage. Animal cap explants were dissected at stage 8 and incubated until stage 10. RT-PCR was performed for the analysis of FoxD5b expression.* **(B)** *The -1336 or -1336-mAP-1 construct was co-injected with AP-1$^{c\text{-}Jun/FosB}$ or DEPC-Q as indicated at the one-cell stage. Animal cap explants were dissected at stage 8, and their luciferase activity was measured at stage 10.* **(C)** *DNBR (0.5ng) and AP-1$^{c\text{-}Jun/FosB}$ (0.5ng) were co-injected at the one-cell stage as indicated. Animal cap explants were dissected at stage 8 and incubated until stage 10. RT-PCR was performed for the analysis of FoxD5b expression.* **(D)** *The -1336 construct was co-injected with DNBR (0.5ng) or AP-1$^{c\text{-}Jun/FosB}$ (0.5ng) as indicated at the one-cell stage. Animal cap explants were dissected at stage 8, and their luciferase activity was measured at stage 10.* **(E)** *AP-1 luc was co-injected with DNBR (2ng) or AP-1$^{c\text{-}Jun/FosB}$ (125pg) as indicated at the one-cell stage. Animal cap explants were dissected at stage 8, and their luciferase activity was measured at stage 10.* **(F)** *DNBR RNA (1ng) was injected into the animal pole region at the one-cell stage. Animal caps were dissected at stage 8 and incubated until stage 10. The western-blot assay was performed with specific anti-phospho c-Jun antibodies (#9261, Cell signaling, MA).* **(G)** *AP-1$^{c\text{-}Jun/FosB}$ mRNAs (1ng) were injected into the one blastomere at the 2 cell stage. Embryos were processed for whole mount* in situ *hybridization with anti-sense probe of FoxD5b at stage 10.* **(H)** *A chromatin immunoprecipitation (ChIP) assay was performed using* Xenopus *embryos at stage 10. The presence of the FoxD5b promoter was detected by PCR in DNA samples obtained from anti-Flag antibody precipitation (Flag), normal IgG antibody precipitation (IgG), and cross-linked chromatin supernatant before immunoprecipitation (Input). RLU, relative luciferase activity. *,* p *value < 0.05, **,* p *value < 0.01; ***,* p *value < 0.001.*

AP-1$^{c\text{-}Jun/FosB}$ complex induced FoxD5b expression in ectodermal explants of *Xenopus* embryos when BMP signaling was inhibited.

Discussion

During the early development of vertebrate embryos, neurogenesis arises at the ectoderm where BMP signaling is inhibited (Dale and Wardle, 1999, Dosch *et al.*, 1997). BMP signaling is required for ectodermal cell differentiation because it acts as instructive signaling, and the inhibition of BMP signaling in animal cap explants leads to neural cells. However, it is still largely unknown how the inhibition of BMP signaling leads to neural cells in ectodermal cell explants. In the present study, we performed microarray analysis to identify the gene profiles involved in early neurogenesis that occurs when BMP signaling is inhibited. The transcription of FoxD5b was increased by the ectopic expression of DNBR in animal cap explants (Table 1, Fig. 1A). The overexpression of FoxD5b induced the expression of neural markers, including SOX2, NeuroD, N-tubulin and NCAM, in animal cap explants (Fig. 1B). A previous study (Schon *et al.*, 2004) reported that FoxD5b is a pseudo-allele gene of FoxD5a,

TABLE 1

PRIMERS USED FOR SERIALLY DELETED REPORTER GENE CONSTRUCTS

Upstream primer	-1336	GGGGTACCTCAAATAATTGACACAAACATGAATG
	-1316	CCGGTACCAATGGCAAGCAGCTCAGGAGTT
	-1196	CCGGTACCCTCTTGAAAGCCCCCCTGTCGTG
	-1016	CCGGTACCATATGCAGAGCTGCTAATAGTC
	-886	CCGGTACCACCATTCTCTTGCATTCCACCA
	-816	CCGGTACCCAGAATTCCAGTTCCCATAATC
	-301	CCGGTACCTTGGATTGCAAGTTAGTGGCTC
Downstream primer		CCGCTCGAGGCTTGGTTGGCAGTAAGTAGTAGAC

TABLE 2

PRIMERS USED FOR RT-PCR ANALYSIS

Marker	Sequence
ODC	U : 5`-GTCAATGATGGAGTGTATGGATC-3` D : 5`-TCCATTCCGCTCTCCTGAGCAC-3`
FoxD5a	U : 5`-GACAGTGAGATGCTGAGTCC-3` D : 5`-GGACTCTGCAGGATAGCCAT-3`
FoxD5b	U : 5`-CAATGCTCCAGCAGACAC-3` D : 5`-TGGTCTCTGCAATAAGTC-3`
Ncam	U : 5`-CACAGTTCCACCAAATGC-3` D : 5`-GGAATCAAGCGGTACAGA-3`
Otx2	U : 5`-GGATGGATTTGTTGCACCAGTC-3` D : 5`-CACTCTCCGAGCTCACTTCTC-3`
NeuroD	U : 5`-GTGAAATCCCAATAGACACC-3` U : 5`-TTCCCCATATCTAAAGGCAG-3`
N-tubulin	U : 5`-ACACGGCATTGATCCTACAG-3` U : 5`-AGCTCCTTCGGTGTAATGAC-3`
Sox2	U : 5`-ACCGGAATTCAGAGAGAGAGAGGCTGTGG-3` D : 5`-ATGCTCTAGACCTTTTTCACATGTGCGAC-3`

TABLE 3

PRIMERS USED FOR THE SITE-DIRECTED MUTAGENESIS OF AP-1 OR A PUTATIVE VENT-1 BINDING SITE IN THE FOX5B REPORTER GENE CONSTRUCT

AP-1(m) F	5`-GTACCTCAAATAATTATCACAAACATGAATGGC-3`
AP-1(m) R	5`-GCCATTCATGTTTGTGATAATTATTTGAGGTAC-3`
Vent-1(m) F	5`-CGATAGGTACCTCGGGTAATTGACACAAACATG-3`
Vent-1(m) R	5`-CATGTTTGTGTCAATTACCCGAGGTACCTATCG-3`

TABLE 4

MICROARRAY GENE EXPRESSION PROFILE OF DNBR-INJECTED ANIMAL CAP EXPLANTS

Probe set ID	Gene ID	Gene	Fold induction
Xl.644.1.S1_at	NM_001098428	*Xenopus* laevis winged helix protein (FoxD5b) mRNA	5.4
Xl.642.1.S1_at	AF162782	*Xenopus* laevis winged helix protein (FoxD5a) mRNA	5.3
Xl.7969.1.S1_at	BC057699	*Xenopus* laevis mRNA for Zic3 protein	5.2
Xl.1946.1.A1_at	BJ080754	Cytochrome P450 retinoid metabolizing protein	4.2
Xl.188.1.S1_at	BC076717	*Xenopus* laevis XlSOX-2 (Sox-2) mRNA	3.6
Xl.22.1.S1_at	BC072222	*Xenopus* mRNA for SOX3 protein	2.1

but our gain-of-function study indicated that FoxD5b is involved in neurogenesis in a similar manner as FoxD5a.

FoxD5b is an early neural-specific gene, which was increased by the inhibition of BMP signaling, and the overexpression of Fox5b increased neural cell differentiation in animal cap explants. To investigate the transcriptional regulation of an early neural-specific gene, FoxD5b, by BMP inhibition, we isolated the promoter region of Fox5b. We examined a reporter assay with the FoxD5b promoter in DNBR-injected animal cap explants. Fox5b promoter activity containing -1336 increased approximately 4 fold when the animal cap explants were injected with DNBR compared with control (Fig. 2C). The reporter assay with serial deletion constructs of the FoxD5b promoter demonstrated that the AP-1 binding site plays a critical role in increasing the FoxD5b transcription induced by the inhibition of BMP (Fig. 2D and 2E).

AP-1 has various functions during *Xenopus* early development. AP-1$^{c\text{-}Jun/c\text{-}Fos}$ induces the expression of the organizer and mesodermal gene, Xbra (Dong *et al.*, 1996, Kim *et al.*, 1998, Lee *et al.*, 2011). In addition, AP-1$^{c\text{-}Jun/c\text{-}Fos}$ regulates Zic3 expression when stimulated with activin (Lee *et al.*, 2004). Under normal circumstances, AP-1$^{JunD/c\text{-}Fos}$ functions in BMP signaling during hematopoiesis (Lee *et al.*, 2012). In the present work, we elucidated the role of AP-1$^{c\text{-}Jun/FosB}$ in DNBR-induced FoxD5b expression. The overexpression of AP-1$^{c\text{-}Jun/FosB}$ increased the promoter activity of FoxD5b, and we confirmed the results of the reporter assay by RT-PCR with c-Jun anti-sense morpholino-oligos. Knockdown of c-Jun reduced FoxD5b expression in DNBR-injected animal cap explants. It is interesting that different combinations of AP-1 components play specific roles in differentiation and signaling.

In the present study, we demonstrated that AP-1$^{c\text{-}Jun/FosB}$ regulated DNBR-induced FoxD5b expression. Our previous work showed that AP-1$^{c\text{-}Jun/c\text{-}Fos}$ regulates Zic3 expression when stimulated with activin (Lee *et al.*, 2004). However, we do not know whether different combinations of AP-1 components regulate specific neural gene differently or whether a single combination of AP-1 plays a more general role in various neural genes including FoxD5, Zic3 and BF-1. For example, AP-1$^{c\text{-}Jun/c\text{-}Fos}$ regulates Zic3 expression when stimulated with activin. However, AP-1$^{c\text{-}Jun/c\text{-}Fos}$ induces the expression of organizer genes including noggin and chordin, which are known as BMP antagonizers. Therefore, we may need to examine whether AP-1$^{c\text{-}Jun/c\text{-}Fos}$-induced Zic3 expression is a direct or indirect component of neurogenesis. In addition, although we showed that AP-1$^{c\text{-}Jun/FosB}$ activity was increased when BMP signaling was inhibited (Fig. 4E), the molecular mechanism regulating AP-1 activity remains unclear. AP-1 activity was usually triggered by an instructive signal. However, the inhibition of BMP signaling triggered AP-1 signaling in this study. The specific AP-1 binding site in the FoxD5b promoter, which responded to the inhibition of BMP signaling, may be a useful tool for elucidating the regulation mechanism of early neurogenesis and the involved signaling network.

Previously, Solter *et al.,* showed that FoxD5a is expressed in the neuroectoderm and open neural plate. In addition, this author addressed that FoxD5a and FoxD5b show identical expression pattern. First, we confirmed the expression pattern of FoxD5b. Spatial and temporal expression pattern of FoxD5b was very similar with the FoxD5a (data not shown). Neilson *et al.,* demonstrated that FoxD5a functions in establishing and maintaining the embryonic neural ectoderm through both inducing genes involved in immature neural ectoderm and suppression genes involved in the transition to a differentiating neural plate. In our research, we found that FoxD5b has amino acid identity of 88% with FoxD5a. Furthermore, over-expression of FoxD5b induced several neural markers similar to FoxD5a (Fig. 1C). The expression of FoxD5a was also reduced by c-Jun morpholino oligos (54/55) (data not shown). Taken together, we expected that FoxD5a and FoxD5b have very similar transcriptional regulation and functions during

early development of *Xenopus* embryos. Interestingly, we found that 5'-flanking region of FoxD5b, which contains AP-1 binding site, was conserved with that of FoxD5a. In this study, our research focused on the transcriptional regulation of FoxD5b by AP-1c-Jun/FosB rather than the functional analysis of FoxD5b during early development. However, further studies woud be necessary to investigate specific roles of FoxD5b during early embryogenesis of *Xenopus* embryos.

The positive response element in FoxD5b promoter that is activated in response to BMP inhibition was examined. However, BMP inhibition results in the reduced expression of BMP target genes, which may have a role in the repression of dorsal and neural genes. The promoter activity of the truncated AP-1 binding site constructs and the mutated AP-1 binding site construct was increased by DNBR in whole embryos (data not shown). This result indicated that the FoxD5b promoter has additional response elements that are modulated by the inhibition of BMP signaling. Because neural genes are usually repressed in the presence of BMP signaling, it will be interesting to elucidate negative response elements in the FoxD5b promoter by BMP target genes including GATA, Msx and Vent family members.

Materials and Methods

Embryo injection and explant culture

Xenopus laevis embryos were obtained by artificial fertilization (Smith and Slack, 1983). Developmental stages were designated according to Nieuwkoop and Faber (Nieuwkoop, 1967). RNA or DNA was injected into the animal pole of embryos at the one-cell stage as described in the figure legends. The animal caps were dissected from the injected embryos at stage 8 and cultured until stage 13 in 67% Leibovitzs L-15 medium (GIBCO/BRL) with BSA (1 mg/ml), 7 mM Tris-HCl (pH 7.5) and gentamicin (50 μ/ml). The cultured explants were incubated at 23°C before harvesting.

Plasmid constructs

Xenopus FoxD5b (GeneBank Accession Number: NM_001098428.1) cDNA was isolated from a *Xenopus* cDNA library and was inserted into the EcoRI and XbaI sites of the pCS2 (+) vector by PCR. The PCR primers that were used were (upstream) 5`-CGGAATTCAAGCTTTAGCCAGGAGTCTG-GAA-3` and (downstream) 5`-GCTCTAGATATGTACAACCAGCAGCCCTC-TAGT-3`. Luciferase reporter genes containing the promoter region of FoxD5b (GeneBank Accession: AJ850136.1) were used to examine the response to the inhibition of BMP signaling. The -1336 bp of the 5`-flanking region of FoxD5b genomic DNA (gDNA) was cloned into a KpnI/XhoI-treated pGL3 basic vector. Serially deleted FoxD5b promoter mutants were made from this -1336 construct by PCR amplification (Table 1). The PCR conditions were as follows: 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C for 20 cycles. The PCR amplification products were digested with KpnI/XhoI and inserted into similarly digested pGL-3 basic vectors.

Morpholino oligos

The antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR). The MO sequences were as follows: MO-Jun54 5'-CTGGAGCTTATGTCAGTGTGA-3'; MO-Jun55 5'-GTAGTTTC-CATCTTTGCGTTCATAC-3'; Cont-MO 5'-CCTCTTACCTCAGTTACAATT-TATA-3'. MO-Jun54 and 55 were designed to bind to complementary sequences found in two types of *Xenopus* c-jun mRNA (Lee *et al.*, 2011) and to prevent the translation of these c-jun mRNA. Morpholino oligo riboside moieties are substituted with nitrogen-containing morpholine moieties and are phosphorodiamidate linked (Summerton and Weller, 1997). The oligos were resuspended in sterile water, and 20 ng of the oligos was injected into each embryo.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

For the qRT-PCR, total RNA was prepared using the TRIzol reagent (Tel-Test, Inc., Friendswood, TX), and cDNA was synthesized using the Superscript pre-amplification system (Invitrogen). The PCR primers and cycling conditions are described at the *Xenopus* Molecular Marker Resource (University of Texas). Additional primers are described in table 2. The PCR reactions were performed with SYBR Premix (Qiagen, Valencia, CA) and a thermal cycler real time system (Qiagen Rotor-Gene-Q, Valencia, CA).

In vitro *transcription*

The FoxD5b mRNAs used for microinjection were produced by *in vitro* transcription. The FoxD5b cDNA was inserted in the pCS2 vector. The cDNA were linearized and used for *in vitro* synthesis of capped mRNA using an *in vitro* transcription kit (Ambion) according to the manufacturer's instructions. The synthetic RNA was quantified by ethidium bromide staining in comparison with a standard RNA.

Luciferase assay

The level of luciferase activity was measured using a luciferase assay system according to the manufacturer's instruction (Promega, Madison, WI). Five or six groups of animal caps (3 animal caps per group) were harvested and homogenized in 30 μl lysis buffer. A luminometer was used to measure 40 μl luciferase substrate with 10 μl whole cell lysate (Promega, Madison, WI). All experiments were repeated at least three times using an independently derived sample set.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described in (Lee *et al.*, 2011), with the following modifications. For ChIP analysis, 1 ng of messenger RNA was injected into the animal pole of embryos at the one-cell stage or two-cell stage, as described in the figure legends. Approximately 100-150 injected embryos were fixed in 1.85% formaldehyde in 0.1 X MBS for 30 min at room temperature. Anti-c-Jun (SC-1694, Santa Cruz, CA) and anti-Flag (M2, Sigma, MO) antibodies were used for immunoprecipitation. A chromatin solution was pre-cleared with Protein A/G PLUS-Agarose beads (SC-2003, Santa Cruz, CA). The embryos were homogenized using a VC 50T (Sonics & Materials Inc.) 20-25 times for 10 sec at amplitude 40. The FoxD5b promoter was assayed by immunoprecipitated DNA using the promoter primers (forward) 5`- GGATAAAGTCAATTGCCCCTC – 3` and (reverse) 5`- GCTCTGCAGCCTACAGTTTAC-3`.

Statistical analysis

Most of the experiments were independently performed more than three times. The data are presented as the means ± SE. A t-test was used to compare groups with the GraphPad Prizm program (GraphPad Software, San Diego, CA).

Site-directed mutagenesis

Mutagenesis was performed using the Site-Directed Mutagenesis Kit (Intronbio, KR) according to the manufacturer's instructions (Table 3).

Whole mount* in situ *hybridization

Embryos were injected with indicated mRNAs, and then processed for whole-mount *in situ* hybridization by using standard methods with antisense probes of FoxD5b.

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