



Ventx1.1 as a Direct Repressor of Early Neural Gene *zic3* in *Xenopus laevis*

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From *Xenopus* embryo studies, the BMP4/Smad1-targeted gene circuit is a key signaling pathway for specifying the cell fate between the ectoderm and neuro-ectoderm as well as the ventral and dorsal mesoderm. In this context, several BMP4/Smad1 target transcriptional factors have been identified as repressors of the neuro-ectoderm. However, none of these direct target transcription factors in this pathway, including GATA1b, Msx1 and Ventx1.1 have yet been proven as direct repressors of early neuro-ectodermal gene expression. In order to demonstrate that Ventx1.1 is a direct repressor of neuro-ectoderm genes, a genome-wide *Xenopus* ChIP-Seq of Ventx1.1 was performed. In this study, we demonstrated that Ventx1.1 bound to the Ventx1.1 response cis-acting element 1 and 2 (VRE1 and VRE2) on the promoter for *zic3*, which is a key early neuro-ectoderm gene, and this Ventx1.1 binding led to repression of *zic3* transcription. Site-directed mutagenesis of VRE1 and VRE2 within *zic3* promoter completely abolished the repression caused by Ventx1.1. In addition, we found both the positive and negative regulation of *zic3* promoter activity by FoxD5b and Xcad2, respectively, and that these occur through the VREs and via modulation of Ventx1.1 levels. Taken together, the results demonstrate that the BMP4/Smad1 target gene, Ventx1.1, is a direct repressor of neuro-ectodermal gene *zic3* during early *Xenopus* embryogenesis.

Keywords: neurogenesis, transcriptional regulation, Ventx1.1, *Xenopus*, *zic3*

INTRODUCTION

BMP4 is a member of the transforming growth factor beta (TGF- β) superfamily which facilitates multiple biological phenomena, including axis patterning, germ-layer specification, differentiation, hematopoiesis, etc. (Dale and Jones, 1999; Munoz-Sanjuan and Brivanlou, 2002). Previous studies have documented BMP4/Smad1 leads the ectoderm and ventral mesoderm formation (VMZ) in *Xenopus* embryos (Dale and Jones, 1999; De Robertis and Kuroda, 2004; Rogers et al., 2009). Blocking of BMP4 signaling by application of dominant-negative BMP receptors (DNBR) also elicits the expression of neural genes, including *foxD5b*, *zic3*, *fgf8a*, *otx2* and *ncam* in animal cap explants (Shim et al., 2005; Yoon et al., 2013; Yu et al., 2016). Studies have shown that BMP4/Smad1 directly induces the expression of Xvent family members, including *ventx1.1* (*PV.1* and *Xvent1b*) and *ventx2.1* (*Xvent2*) in animal cap explants of *Xenopus* embryos (Henningfeld et al., 2002; Lee et al., 2002; Lee et al., 2011). Ventx1.1 is a homeobox transcription factor that represses the expression of organizer-specific genes, including *gsc*, *chordin*, and *noggin*, resulting in the headless phenotype in *Xenopus* embryos (Ault et al., 1996; Hwang et al., 2002; Hwang et al., 2003). In addition, overexpression of Ventx1.1 causes embryos ventralization and ventral mesoderm formation in *Xenopus* embryos. Overexpressed Ventx1.1 also inhibits the expression of early and late neural genes that include *zic3*, *foxD5b* and

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ncam in animal cap explants of *Xenopus* embryos, leading to neural inhibition (Yoon et al., 2014b). Furthermore, morpholino-based knockdown of *ventx1.1* increasing the expression of neural and dorsal genes, including *zic3*, *foxD5b*, *chordin*, *noggin*, *gsc*, *ncam* and *Xngnr* in animal cap explants, resulting in organizer expansion and neural induction in *Xenopus* embryos (Kumar et al., 2018; Yoon, Kim et al., 2014b). The detailed molecular mechanism of Ventx1.1-mediated direct inhibition of early neural genes during embryonic development of *Xenopus*, however, still awaits study.

Zic3 has been documented as being a member of the zinc finger transcriptional activator and one of the earliest neural genes actively involved in neural tissue specification, including for neural crest and neural plate formation (Houtmeyers et al., 2013; Nakata et al., 1998). A recent study demonstrated Activin/Smad2/3 positively regulating *zic3* expression in an AP-1^{c-jun/c-fos}-dependent manner (Lee et al., 2004) and that the ectopic expression of AP-1 induces *zic3* expression to trigger neurogenesis in animal cap explants of *Xenopus* embryos. Previous study has shown the ectopic expression of Zic family members, Zic1-3, leading to expression of the neural crest and neuro-ectoderm specific genes, including *en2*, *Xngnr*, *slug*, *N-tubulin* and *twist* in animal cap explants of *Xenopus* embryos, and promoting neural crest specification and neuro-ectoderm formation (Nakata, Nagai et al., 1998).

In the present study, Ventx1.1 inhibited the Engrailed repressor domain-fused FoxD5b (FoxD5b-En)-induced mRNA expression level of early and late neural genes, including *zic3*, *foxD5b*, *ncam* and *NeuroD* for whole embryos and animal cap explants. In order to explore the possibility of Ventx1.1 as a direct repressor of a key early neuro-ectoderm gene, *zic3*, a genome-wide *Xenopus* ChIP-Seq analysis of Ventx1.1 show that Ventx1.1 directly binds to Ventx1.1 response cis-acting element 1 and 2 (VRE1 and VRE2) within the 5'-flanking upstream region of *zic3* and represses its transcription. Reporter gene assay demonstrated that Ventx1.1 reduces the relative promoter activity of serially-deleted promoter constructs of *zic3* in *Xenopus* embryos. Site-directed mutagenesis provide evidence that *zic3* promoter construct contains the Ventx1.1 response elements, VRE1 and VRE2, which directly inhibit *zic3* transcription activation in *Xenopus* embryos. Additionally, it is shown that FoxD5b indirectly increases the relative promoter activity of *zic3* by inhibiting the endogenous expression of *ventx1.1* while Xcad2 inhibits the relative promoter activity of *zic3* by inducing *ventx1.1* expression in *Xenopus* embryos. Collectively, the results suggest that Ventx1.1 is a direct repressor of the transcription of *zic3* and inhibits neurogenesis as one of the key regulatory

molecules of BMP4/Smad1 signaling in ectoderm and VMZ during the embryonic development of *Xenopus*.

MATERIAL AND METHODS

Ethics statement

An approval by the Institutional Animal Care and Use Committee (IACUC) is currently not required for the experimental use of amphibians or reptiles in Korea. However, all our research members attended both the educational and training courses for appropriate care and usage of experimental animals at our institutions. Adult *X. laevis* were grown in 12 hr light/dark (LD 12:12 hr) cycles at 18°C according to the guidelines of Institutes of Laboratory Animal Resources that are tasked for laboratory animal maintenance.

DNA and RNA preparation

All mRNA used for this study were synthesized by linearizing the target vectors with the appropriate restriction enzymes, including FoxD5b-En: T7, BamHI, Xcad2: Sp6, Asp718 and Flag-Ventx1.1: Sp6, SacII. Each vector was linearized with the appropriate restriction enzyme and used for in-vitro transcription using the MEGA script kit according to manufacturer's instructions (Ambion, USA). Synthetic mRNAs were quantified by a spectrophotometer at 260/280nm (SpectraMax, Molecular Devices, USA).

Cloning of *zic3* genomic DNA

Cloning of *zic3* genomic DNA (gDNA) into the pGL3-Basic plasmid (Promega, USA) was performed as described by (Lee et al., 2004).

Zic3 promoter constructs

The 1.8 kb of 5'-flanking region of reporter construct was further sub-cloned into the pGL3-basic plasmid (Promega) by using specific restriction sites of KpnI/HindIII. Serially-deleted *zic3* promoter constructs were generated and sub-cloned into a pGL3-basic plasmid by PCR amplification (Table 1).

Embryo injection and explants culture

Xenopus laevis were obtained from the Korean *Xenopus* Resource Center for Research. *Xenopus* embryos were injected after in vitro fertilization of oocytes that was induced by injection of 500 units of human chorionic gonadotropin (Sigma, USA). RNAs were injected into the animal pole at the one or 2-cell stage embryos and cultured in 30% Marc's Modified Ringer's (MMR) solution. The animal caps (AC) were then dissected from injected and un-injected embryos at stage 8.0-8.5 and incubated in 1X L-15 growth medium

Table 1. Primers used for serially-deleted reporter gene constructs

Primers	Primer Name	Sequences (5' → 3')
Upstream primers	-1805	GCGGGTACCTTTTTTACACTTTGTAGGTAT
	-910	GCGGGTACCTTATGCTGCACGAAAGG
	-517	GCGGGTACCCTCATGAACTGCAAAGCTG
	-297	GCGGGTACCAACATCTTTCCACAATCCAT
Downstream primer		GCGAAGCTTCTCTCCAAGTGAATATGTC

Table 2. Primers used for RT-PCR amplification

Gene Name	Sequences	Annealing Temp (°C)	Cycles
Zic3	F5'-TTCTCAGGATCTGAACACCT-3' R5'-CCCTATAAGACAAGGAATAC-3'	45	28
FoxD5b	F5'-ACTCTATCAGGCACAACCTGTC-3' R5'-GGTCTGTAGTAAGGCAGAGAGT-3'	50	30
Xbra	F5'-GGATCGTTATCACCTCTG-3' R5'-GTGTAGTCTGTAGCAGCA-3'	57	25
EF1 α	F5'-CCTGAATCACCCAGGCCAGATTGTG-3' R5'-GAGGGTACTCTGAGAAAGCTCTCCACG-3'	57	19
NCAM	F5'-CACAGTCCACCAAATGC-3' R5'-GGAATCAAGCGGTACAGA-3'	57	29
Xngnr1	F5'-GGATGGTGCTGCTACCGTGCAGTACC-3' R5'-CAAGCGCAGAGTTCAGGTTGTGCATGC-3'	65	30
Otx2	F5'-GGATGGATTGTTGCACCAGTC-3' R5'-CACTCTCCAGCTCACTTCTC-3'	57	27
Xk81	F5'-TGGTGTGAACAAGTGCAGG-3' R5'-ACCTCTCGACAATGGTCTT-3'	57	25
Krox20	F5'-AACCGCCCCAGTAAGACC-3' R5'-GTGTCAGCCTGTCTGTTAG-3'	57	28
NeuroD	F5'-GTGAAATCCCAATAGACACC-3' R5'-TTCCCCATATCTAAAGGCAG-3'	57	28

(Gibco/Thermo Fisher, USA) until stage 11 or stage 24 in preparation for RT-PCR.

RNA isolation and RT-PCR

Ventx1.1 mRNA (500 pg/embryo) and FoxD5b-En mRNA (280 pg/embryo) were injected into the animal pole at the one or 2-cell stage of *Xenopus* embryos and cultured in 30% MMR solution. Animal caps were then dissected from the injected and un-injected embryos and incubated until stage 11 or 24 in 1X L-15 growth medium. Total RNA was isolated from whole embryos and animal cap explants using RNA-Bee reagent and following the manufacturer's instructions (Tel-Test, USA) and it was treated with DNase I to remove genomic DNA contamination. RT-PCR was performed with Superscript II (Invitrogen, USA) as described by the manufacturer and with 2 μ g total RNA per reaction. PCR was performed according to the following conditions: 30 s at 94°C, 30 s at each annealing temperature, 30 s at 72°C, and 20-30 cycles of amplification (Table 2).

Luciferase assays

Relative promoter activities were measured using a luciferase assay system according to the manufacturer's instructions (Promega). Five different groups of embryos (3 embryos per group) were harvested and homogenized in 10 μ l lysis buffer per embryo. Embryo homogenates at 10 μ l each were assayed with 40 μ l luciferase substrate and the reporter gene activity was read by an illuminometer (Berthold Technologies, Germany). All experiments were repeated at least three times for independently derived sample sets.

Site-directed mutagenesis

Mutagenesis was performed by a site-directed mutagenesis kit (Muta-Direct, iNtRON Biotechnology, Korea) using the primer oligonucleotides in accordance with the manufacturer's instructions (Table 3).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed as

Table 3. Primers used for site-directed mutagenesis gene constructs

Mutated Site	Primer Name	Sequence
mVRE1	Zic3mVRE1	F5'-AATGTGTGGGAAAATA GGGGTTTCATTCAAGTATC3' R5'-GATACTTGAATGAAC CCTATTTCCACACATT-3'
mVRE2	Zic3mVRE2	F5'-TTACAATTAGCCATC GGGCTCTTTGCCTTTTAT-3' R5'-ATAAAAGGCAAAGAG CCGATGGCTAATTGTAA-3'

Table 4. Primers used for ChIP-PCR assay

Primer Name	Sequences	Annealing Temp (°C)	Cycles
<i>Zic3</i> -VRE1-ChIP	5'-TTCTCAGGATCTGAACACCT-3' R5'-CCCTATAAGACAAGGAATAC-3'	52	30
<i>Zic3</i> -VRE2-ChIP	5'-CTTGATCAGATTTTATGTTTC-3' R5'-TTTGGAAATAATGCATTGTC-3'	46	25

described (Blythe et al., 2009). Embryos were injected at the one-cell stage with mRNA encoding 3xFlag-Ventx1.1 (500 pg/embryo). Injected embryos were collected at stage 11 (100 embryos/sample) and processed according to the protocol. Anti-Flag monoclonal antibodies (F-1804, Sigma) or normal mouse IgG (SC-2025, Santa Cruz Biotechnology, USA) were then added to the cell lysates to immunoprecipitate the chromatin. ChIP-PCR was performed with the immune-precipitated chromatin using *zic3* promoter region primers for VRE1 (163 bps) and VRE2 (245 bps), respectively. These primers are shown in Table 4.

Nucleotide sequence accession number

The *zic3* (Accession number; AF179297) promoter sequence has been submitted to GenBank.

Statistical analysis

Data were analyzed by unpaired two-tailed Student's t test using GraphPad Prism4 (GraphPad, USA). Asterisks designations are ** for $P \leq 0.01$ and *** for $P \leq 0.001$. n.s. denotes non-significance.

RESULTS

Ectopic expression of Ventx1.1 represses the expression of early and late neural genes in *Xenopus* animal cap explants and whole embryos

Ventx1.1 is a well-known transcriptional repressor of the Xvent family and is one of the direct downstream targets of BMP4/Smad1 signaling (Lee, Lee et al., 2011). Studies have shown that ectopic expression of Ventx1.1 strongly inhibits neurogenesis and organizer expansion in *Xenopus* embryos. Overexpression of Ventx1.1 inhibits head-and neural-inducing factors including *gsc*, *chordin*, *noggin*, *zic3*, *foxD5b* and *cerberus*, resulting in headless phenotype and neural inhibition (Hwang, Lee et al., 2003; Yoon et al., 2014a). In the present study, RT-PCR shows that FoxD5b-En-induced expression of early and late neural genes could be inhibited by Ventx1.1 expression. Ventx1.1 mRNA co-injection with FoxD5b-En significantly reduced the FoxD5b-En-induced expression of early and late neural genes, including *zic3* and *foxD5b* (early) at stage 11 (Figs. 1A and 1C) and *ncam*, *NeuroD* and *Xngnr1* (late) at stage 24 (Figs. 1B and 1D) both in

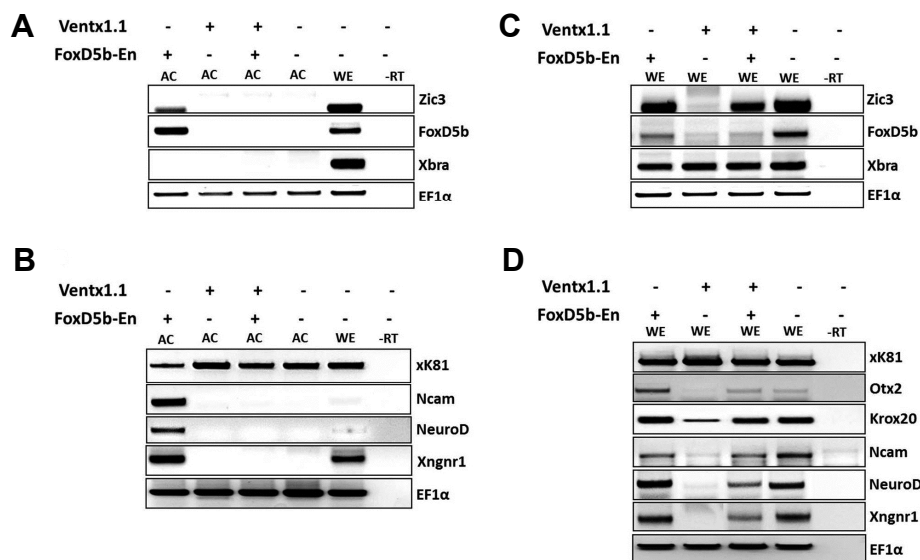


Fig. 1. Ventx1.1 represses early and late neural genes in *Xenopus* whole embryo and animal cap explants. Ventx1.1 (500 pg/embryo) mRNA was co-injected with and without FoxD5b-En mRNA (280 pg/embryos) at the one-cell stage, followed by dissection of the animal cap explants (AC) at stage 8 and harvesting at stage 11 and 24 respectively. The relative gene expressions were analyzed by RT-PCR: (A and C) Ventx1.1 reduced the FoxD5b-induced expression of *zic3* and *foxD5b* in both animal cap explants (A) and whole embryos (WE) (B) at stage 11. (B and D) Ventx1.1 reduced the FoxD5b-En-induced expression of *ncam*, *Xngnr* (pan-neural markers) and *NeuroD* (neuronal differentiation marker) while induced the expression of *xK81* (keratin marker) at stage 24. *EF1α* loading control, non-injected animal caps (negative control), whole embryo (WE) (positive control), control reaction without reverse transcriptase (-RT).

animal cap explants (Figs. 1A-1B) and in whole embryos (Figs. 1C-1D). On the other hand, neuro-ectodermal gene (FoxD5b-En) injection reduced the expression of epidermal gene, *xk81* in animal cap explants and in whole embryos of *Xenopus* (Figs. 1B and 1D; 1st lines). These results collectively indicated Ventx1.1 negatively regulating the neurogenesis in *Xenopus* embryos. Ventx1.1 inhibited the *zic3* expression which was induced by FoxD5b-En in *Xenopus* embryos. The results suggested that Ventx1.1 may directly inhibit the transcription of early neural genes, including *zic3* and *foxD5b*, causing the neural inhibition in *Xenopus*. These results support the previous studies that Ventx1.1 inhibits neuro-ectoderm formation and organizer expansion, resulting in the headless phenotype in *Xenopus* embryos (Hwang et al., 2002; 2003).

Genome-wide *Xenopus* ChIP-Seq analysis of Ventx1.1 identifies Ventx1.1 response cis-acting elements (VRE) on *zic3* promoter

Previous studies have demonstrated that Ventx1.1 is one of the endogenous neural inhibitors of the Xvent family in *Xenopus* embryos (Kumar et al., 2018; Yoon et al., 2014a).

Our present study reiterated the neural repression activity of Ventx1.1 in *Xenopus* (Fig. 1). To investigate the role of Ventx1.1 as a neural repressor and its genome-wide targets in *Xenopus* embryos, we performed a genome-wide Ventx1.1 ChIP-Seq analysis during the gastrula stage after injection of Ventx1.1 mRNA at the one-cell stage embryos. ChIP-Seq results showed that Ventx1.1 bound within the 5'-flanking upstream region of specific neural and dorsal genes, including *zic3*. ChIP-Seq analysis of coverage plot within the 5'-flanking upstream region of *zic3*, where Ventx1.1 protein was bound, showed that Ventx1.1 bound to the *zic3* promoter region on more than one putative consensus site in *Xenopus* embryos (Fig. 2B), but not for all the putative consensus binding sites (Fig. 2A). The results indicated that *zic3* promoter may contain more than one functionally active Ventx1.1 binding response element. Ventx1.1 ChIP-Seq peak-calling and coverage plot analysis showed Ventx1.1 binding to 4 different regions within the 5'-flanking upstream region of *zic3* (Fig. 2B). The putative Ventx1.1 binding sequences within the *zic3* promoter using chromosome 8S (Chr8S) were then analyzed (Fig. 2C). The promoter sequences of *zic3* Chr8S were compared with those of Chr8L

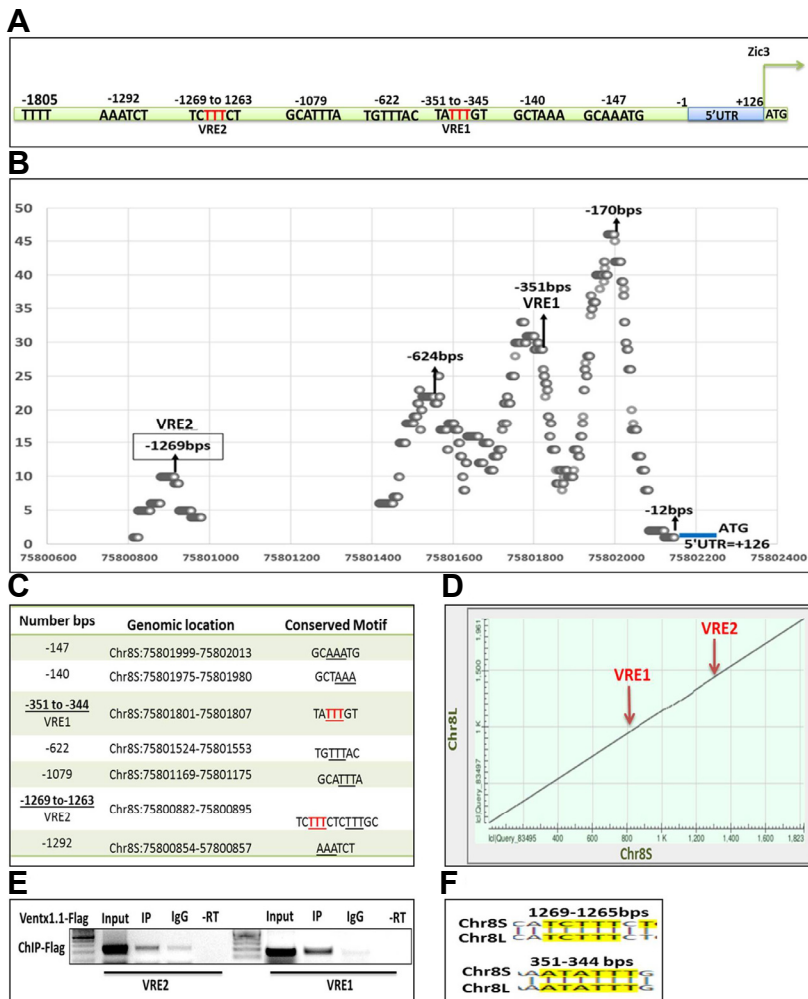


Fig. 2. Genome-wide *Xenopus* ChIP-Seq of Ventx1.1 identifies two Ventx1.1 response cis-acting elements (VRE1 and VRE2) on the *zic3* promoter. (A) Putative Ventx1.1 response consensus binding elements (VREs) are presented within the upstream promoter region of *zic3* gene. (B) ChIP-Seq coverage plot of 3Flag-Ventx1.1 on the *zic3* promoter. (C) Putative VRE sequences and their locations within the *zic3* promoter. (D) This plot is showing the similarity in structure between Chr8S and Chr8L of *zic3* promoter with the VRE1 and VRE2 locations marked. (E) In the ChIP assay, 3Flag-Ventx1.1 construct (500 pg/embryos) was injected at the one-cell stage and the embryos were harvested at stage 11 in 30% MMR solution. Anti-Flag antibody was used to immune-precipitate the endogenous *zic3* promoter region. VRE1 and VRE2 were measured by PCR with specific *zic3* promoter primers. (F) Consensus binding motifs of VRE1 and VRE2 are fully matched in both Chr8S and Chr8L of the *zic3* promoter regions.

and were found to have the promoter sequences of Chr8S and Chr8L having an overall similarity of 91% (Fig. 2D). To verify the direct binding of Ventx1.1 within the *zic3* promoter regions, we injected Flag-Ventx1.1 mRNA at the one-cell stage of *Xenopus* embryos and performed the ChIP-PCR assay using gastrula embryos. Results showed that Ventx1.1 directly bound to within the distal region of the endogenous *zic3* promoter at two different Ventx1.1 response elements (VRE1 and VRE2) in *Xenopus* embryos (Fig. 2E). The two VRE sequences targeted for ChIP-PCR within the *zic3* promoter were exactly conserved in Chr8S and Chr8L (Fig. 2F). Taken together, genome-wide *Xenopus* ChIP-Seq analysis of Ventx1.1 led to identification of Ventx1.1 response cis-acting elements (VREs) within the *zic3* promoter.

Site-directed mutagenesis of VRE1 and VRE2 on *zic3* promoter completely abolished the Ventx-1.1-mediated repression of *zic3* promoter

To identify the functionally active Ventx1.1 binding response

elements (VREs) within the *zic3* promoter region, we cloned the 1.8 kbs long 5'-flanking region of *zic3* with respect to the putative transcription initiation site (TSS) and performed a reporter gene assay at the embryonic stage 11. Already shown, Ventx1.1 directly bound to the *zic3* promoter and abolished the mRNA expression of *zic3* and *foxD5b* in animal caps explants and whole embryos. As such, we then asked whether Ventx1.1 would indeed inhibit the relative promoter activity of the isolated *zic3* promoter. We examined the *zic3*(-1805) reporter construct and its serially-deleted promoter constructs (Fig. 3A) with and without Ventx1.1 at the one-cell stage. The results showed Ventx1.1 significantly reduced (by 4 to 5-fold) the relative reporter activities of serially-deleted promoter constructs of *zic3*(-1805, -910 and -517) when compared to that of the *zic3* promoter construct in the absence of Ventx1.1 in *Xenopus* embryos (Fig. 3B, bar 1-6). The relative reporter activity of *zic3*(-517) promoter construct (Fig. 3B, bar 5-6) was similarly reduced by Ventx1.1 when compared to those of the longer

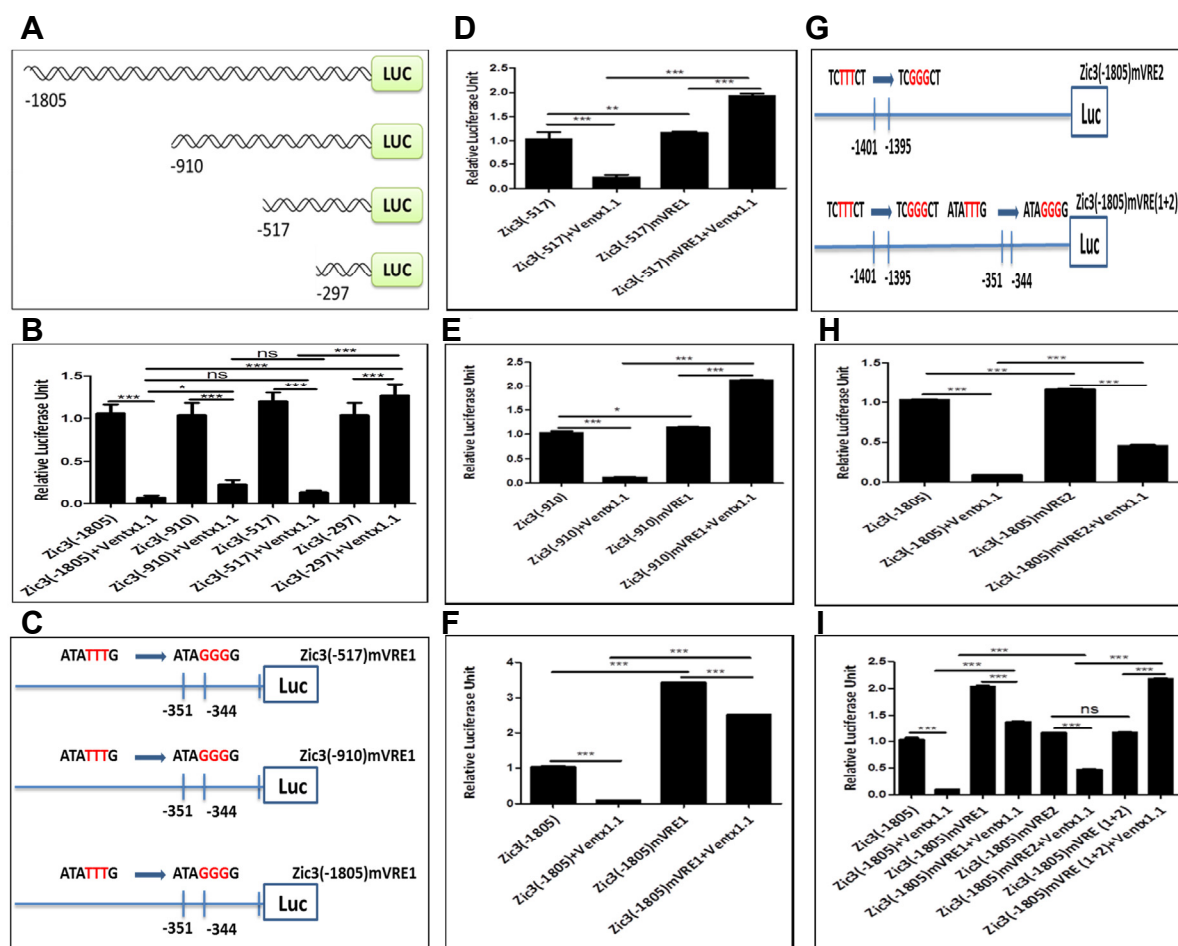


Fig. 3. Site-directed mutagenesis of VRE1 and VRE2 within the *zic3* promoter-reporter construct abolishes the repressional activity of Ventx1.1 on the reporter. (A) Schematic representation of serially-deleted *zic3* promoter constructs. (A-I) Different serially-deleted *zic3* (40 pg/embryos) promoter constructs were co-injected with and without Ventx1.1 (500 pg/embryos) at the one-cell stage and grown until stage 11 to measure the relative promoter activity. The data are shown as mean \pm S.E. of the values from at least three independent experiments. Differences were considered significant at $P < 0.05$.

constructs, *zic3*(-1805 and -910) (Fig. 3B, bar 1-4). For the *zic3*(-297) promoter construct, however, the Ventx1.1-mediated reduction in the relative promoter activity of *zic3*(-297) was completely abolished (Fig. 3B, bar 7-8).

Mapping of the *zic3* promoter nucleotide sequences between -517 and -297 indicated that the *zic3*(-517) promoter construct contains one putative Ventx1.1 consensus sequence (VRE1) within the -351 bps to -344 bps from the putative TSS (Fig. 3C). To verify the VRE1 consensus sequence as an active cis-acting element, we performed site-directed mutagenesis (ATA**TTT**G to ATA**GGG**G) of the putative VRE1 consensus sequence in *zic3*(-1805, -910 and -517) promoter constructs (Fig. 3C). We then tested the relative promoter activity of wild-type and mutated VRE1 promoter constructs *zic3*(-1805, -910, and -517) with and without Ventx1.1. Results showed Ventx1.1-mediated reduction in the relative promoter activity of *zic3*(-910 and -517) promoter constructs being completely abolished with *zic3*(-910)mVRE1 and *zic3*(-517)mVRE1 promoter constructs (Figs. 3D-3E) while Ventx1.1-mediated reduction in the relative promoter activity of *zic3*(-1805) promoter construct being significantly, but not completely, preserved in *zic3*(-1805)mVRE1 promoter constructs (Fig. 3F). These results collectively confirmed that VRE1(-351 bps to -344 bps) acts as a direct cis-acting response element for Ventx1.1 binding and plays a significant role in inhibiting the *zic3* transcription activation. Since Ventx1.1 decreased the relative promoter activity of *zic3*(-1805)mVRE1 construct, we reasoned that *zic3*(-1805) may also contain another VRE within the upstream region of *zic3*(-910) as Ventx1.1-mediated reduction in the relative promoter activity of *zic3*(-910) was completely abolished in *zic3*(-910)mVRE1 (Fig. 3F). We then mapped the nucleotide sequences between *zic3*(-1805 to -910) and found 3 putative VRE consensus binding sequences upstream of the putative *zic3* TSS (Figs. 3B and 3C). The peak-calling analysis of Ventx1.1 ChIP-Seq indicated Ventx1.1 directly bound within the upstream region of *zic3* promoter on one putative consensus sequence located within the -1269 bps to -1263 bps upstream of *zic3*(-910) (Figs. 2 and 3G). We next examined whether the putative VRE consensus binding site2 (VRE2) is also required for Ventx1.1-mediated repression of *zic3* transcription in *Xenopus* embryos. The VRE2 site was found in Ventx1.1 ChIP-Seq peak-calling analysis for coverage fold (≥ 10 coverage fold) (Figs. 2B and 2C). We next performed site-directed mutagenesis within the (-1269 bps to -1263 bps) and generated the VRE2-mutated *zic3*(-1805)mVRE2 and doubly-mutated *zic3*(-1805)mVRE(1+2) promoter constructs (Fig. 3G). These constructs were then examined for their relative promoter activity in the context of presence and absence of Ventx1.1. Results showed that *zic3*(-1805)mVRE2 construct restored the Ventx1.1-mediated reduction in the relative promoter activity of *zic3*(-1805), and *zic3*(-1805)mVRE2 still significantly sustained Ventx1.1-mediated reduction (about 2.5-fold) in the relative promoter activity. This compared with reduction by Ventx1.1 of 10-fold in the relative promoter activity of wild-type *zic3*(-1805) promoter construct compared to that of *zic3*(-1805) alone (Fig. 3H). This indicated that VRE2 also significantly contributes to Ventx1.1-mediated repression of *zic3* transcription activation.

In summary, both cis-acting elements of VRE1(-351 bps to -344 bps) and VRE2(-1269 bps to -1263 bps) seem to contribute to Ventx1.1-mediated repression of neural gene *zic3* transcription. To confirm this, we tested the wild-type *zic3*(-1805) and mutated constructs *zic3*(-1805)mVRE1, *zic3*(-1805)mVRE2, and the doubly-mutated *zic3*(-1805)mVRE(1+2) version with and without Ventx1.1 in *Xenopus* embryos. The results showed that Ventx1.1-mediated reduction in the relative reporter activity of wild-type *zic3*(-1805) promoter was completely restored in doubly-mutated *zic3*(-1805)mVRE(1+2) (Fig. 3I, 7-8th bar). Actually, Ventx1.1 significantly increased the relative promoter activity of doubly-mutated *zic3*(-1805)mVRE(1+2) promoter construct (Fig. 3I). From these experiments, it was concluded that *zic3*(-1805) promoter contains two VRE cis-acting response elements. VRE1 is within the (-351 bps to -344 bps) region while VRE2 is located within the (-1269 bps to -1263 bps) 5'-flanking upstream region of *zic3* from its putative TSS.

Xcad2 and FoxD5b-En regulate *zic3* transcription in a Ventx1.1-dependent manner in *Xenopus* embryos

Previously, FoxD5b-En increased *zic3* expression in both animal cap explants and whole embryos as shown via RT-PCR (Fig. 1). To examine whether FoxD5b-En would also increase the relative reporter activities of various *zic3* promoter constructs, we co-injected the serially deleted *zic3* promoter constructs with and without FoxD5b-En at the one-cell stage and measured their relative reporter activities in *Xenopus* embryos. Results showed that FoxD5b-En increased the relative promoter activity of serially-deleted *zic3* promoter constructs from 1.5 to 2.5-fold compared to the *zic3* promoter construct alone and depending on the construct (Fig. 4A). The results indicated that *zic3*(-517) promoter construct may contain FoxD5b binding response elements (FRE) which positively regulate *zic3* transcriptional activation in *Xenopus* embryos. The *zic3*(-517) promoter construct also contains a direct Ventx1.1 binding response elements1 (VRE1) within the -351 bps to -344 bps region of *zic3* promoter. Our unpublished results have shown that FoxD5b directly inhibits *ventx1.1* transcription in *Xenopus* embryos. Thus, we asked whether FoxD5b increases *zic3* promoter activity in a Ventx1.1-dependent manner and examined the VRE1-mutated *zic3*(-517)mVRE1 promoter construct with and without FoxD5b-En. Results showed that FoxD5b-En-mediated induction in the relative promoter activity of *zic3*(-517) promoter construct was completely abolished in *zic3*(-517)mVRE1 (Fig. 4B).

Previous study has demonstrated that Xcad2 is one of the positive regulators of BMP4 signaling and a homeobox transcriptional activator which induces the expression of *bmp4* and *ventx1.2* while it inhibits the expression of *gsc* and *chordin* in order to inhibit embryo dorsalization in *Xenopus* embryos (Levy et al., 2002; Pillemer et al., 1998). Since ectopic expression of Xcad2 inhibits organizer specific gene including *gsc* and *chordin*, we asked whether Xcad2 negatively regulates *zic3* transcription. The reporter activity of *zic3*(-517) was examined with and without Xcad2 in *Xenopus* embryos. Results showed that Xcad2 reduced the relative promoter activity of *zic3*(-517) (Fig. 4C, 1st and 2nd

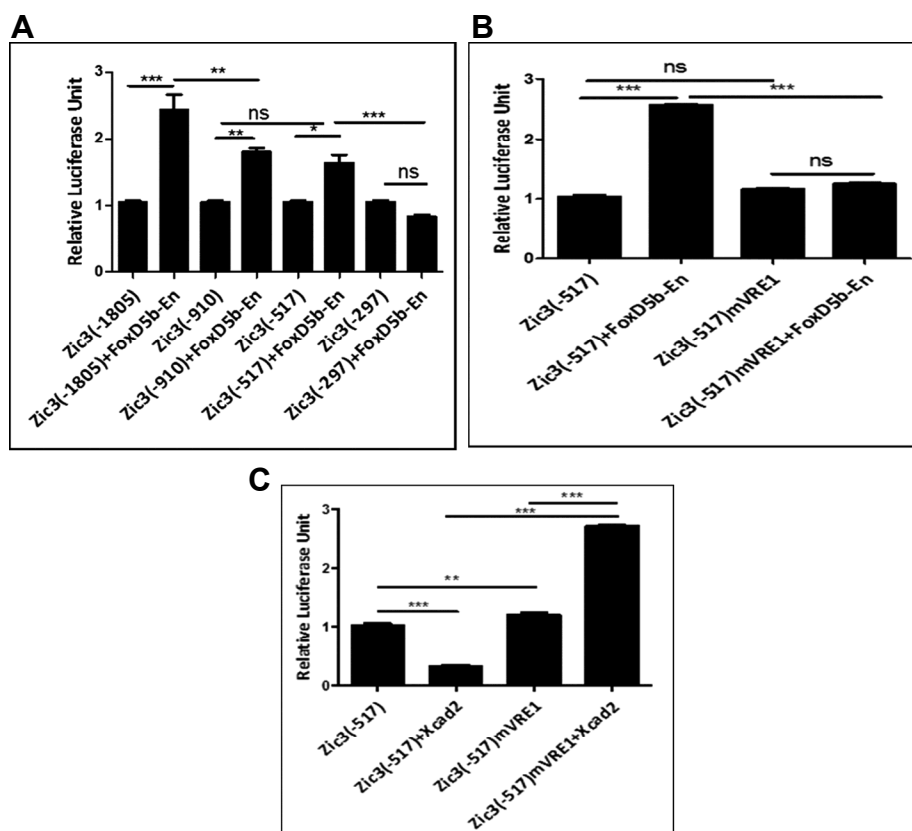


Fig. 4. VRE1 mutation partially abolishes negative and positive effects of Xcad2 and FoxD5b-En, respectively. Different serially-deleted *zic3*(40 pg/embryos) promoter constructs were co-injected with and without FoxD5b-En (280 pg/embryos) at the one-cell stage and grown until stage 11 to measure the relative promoter activity. (A) FoxD5b-En increased the relative promoter activity of *zic3*(-1805) promoter region compared to *zic3*(-1805) alone. (B) *zic3*(-517) and *zic3*(-517)mVRE1 promoter constructs were co-injected with and without FoxD5b-En in separate groups. The FoxD5b-mediated induction in the relative promoter activity of *zic3*(-517) was abolished in *zic3*(-517)mVRE1. (C) *zic3*(-517) and *zic3*(-517)mVRE1 promoter constructs were co-injected with and without Xcad2 in separate groups. The Xcad2-mediated reduction in the relative promoter activity of *zic3*(-517) was abolished in *zic3*(-517)mVRE1 reporter construct. The data are shown as mean \pm S.E. of the values from at least three independent experiments. Differences were considered significant at $P < 0.05$.

bar). However, Xcad2-mediated reduction in the relative promoter activity of *zic3*(-517) promoter construct was completely abolished in *zic3*(-517)mVRE1 (Fig. 4C, 3rd and 4th bar). These results point to having FoxD5b and Xcad2 regulate *zic3* transcription in a *ventx1.1*-dependent manner. From these observations, *Ventx1.1* as one of the negative regulators of neurogenesis directly inhibits the *zic3* transcription in *Xenopus* embryos. In this light, *Ventx1.1* mediates its inhibition on the *zic3* promoter via two *Ventx1.1* consensus binding response elements within the 5'-flanking distal region of *zic3*, namely VRE1 (-351 bps to -344 bps) and VRE2 (-1269 bps to -1263 bps).

DISCUSSION

As an immediate early transcription factor (TF) of BMP4/Smad1 signaling, *ventx1.1* is one of the direct binding repressors acting on the promoter of early neuro-ectoderm gene *zic3*

BMP4/Smad1 is a crucial signaling circuit determining the fate of ectoderm cells for ectoderm and neuro-ectoderm as well as for ventral and dorsal mesoderm in the early embryogenesis of vertebrates (Ault et al., 1996; Dale and Jones, 1999; Hwang et al., 2003). Both gradients of BMP4/Smad1 from ventral to dorsal mesoderm and from ectoderm to endoderm are important for primordial germ layer specification of mid-blastula embryos (Cha et al., 2008; Maeno et al., 1996; Sasai, 1998). A high concentration of instructive BMP4 signal is required to induce the ventral mesoderm and ectoderm (Maeno et al., 1996; Suzuki et al., 1997a; Wilson et al., 1997), but on the other hand, inhibition of BMP4 signal is crucial for generating the dorsal mesoderm (Spemann organizer) for blastula embryos and the neuro-ectoderm for the gastrula embryos (Delaune et al., 2005; De Robertis and Kuroda, 2004; Sasai et al., 1995; Xu et al., 1995). At the mid-blastula stage, a BMP4/Smad1 signal starts to induce transcription of a set of immediate early genes (maternal to zygotic transition genes), including

ventx1/2, *gata1/2*, and *msx1*, the transcription factors in the ventral side of embryos. Our previous studies have shown that Ventx1.1 is one of the direct targets of BMP4/Smad1 signaling and inhibits neurogenesis and organizer specific genes in *Xenopus* embryos. These studies demonstrated that *ventx1.1* functions as a repressor of neuro-ectoderm formation under various conditions (Kumar et al., 2018; Hwang et al., 2002; 2003; Yoon et al., 2014a; 2014b). However, an observation of the direct inhibition of early neural genes by Ventx1.1 has been elusive and not reported.

From the recent completion of the *Xenopus laevis* genome project (Session et al., 2016), we have started the investigation of various gene regulatory networks underlying germ layer specification at a genome-wide scale. Genome-wide target validation experiments, including ChIP-Seq analysis, can now be performed for mapping of various transcription factors directly binding and modulating various promoters and for uncovering various transcriptional regulatory networks relevant to various developmental stages. In the present study, genome-wide Ventx1.1 ChIP-Seq assay indicated that during gastrula of *Xenopus laevis*, neural gene *zic3* is one of the direct targets of Ventx1.1 and that Ventx1.1 directly binds to the 5'-flanking region of *zic3* at more than one functional site (Figs. 2A-2C).

Previously, it was reported that downstream target genes of BMP4/Smad1 such as *gata-1b*, *msx1* and *Dlx1* promote epidermal differentiation and inhibit early neurogenesis (Feledy et al., 1999; Suzuki et al., 1997b; Xu et al., 1997). However, a direct binding of those neural repressors on cis-acting elements within the promoter regions of early neural genes has not been reported. In the present study, genome-wide ChIP-Seq analysis of Ventx1.1 for *Xenopus laevis* identified Ventx1.1 cis-acting elements on the *zic3* promoter region. In addition, Ventx1.1 was found to be one of the negative regulators of the early neuro-ectoderm gene, *zic3*. The *zic3* promoter contains two Ventx1.1 consensus binding response elements, VRE1 (-351 bps to -344 bps) and VRE2 (-1269 bps to -1263 bps) which locate within the 5'-flanking region of *zic3* (Figs. 1-3). ChIP-Seq peak-calling analysis indicated four major binding sites for Ventx1.1. In this work, we assigned two of those four binding sites as together being able to lead to complete inhibition of *zic3* promoter as shown via various reporter constructs in *Xenopus* embryos (Fig. 3I).

Ventx1.1 is a key molecule in the positive and negative circuit for ectoderm and neuro-ectoderm specification

Ventx1.1 as one of the direct downstream targets of BMP4/Smad1 signaling directly represses expression of *zic3* and via direct binding within the endogenous promoter region of *zic3* (Fig. 2E). FoxD5b-En, on the other hand, increases the relative promoter activity of *zic3* (Fig. 4A). A previous study using microarray analysis showed that FoxD4L1/D5 inhibits ventral gene expression, including *bmp4* and *dlx1* (Yan et al., 2010). As FoxD5b inhibits *bmp4* expression that leads to inhibition of *ventx1.1* expression, it was interesting to evaluate Ventx1.1 involvement in FoxD5b-mediated induction of *zic3* expression. We reasoned that as FoxD5b-En induces *zic3* expression, this may at least be par-

tially through the inhibition of Ventx1.1. The site-directed mutagenesis construct of *zic3*(-517)mVRE1 also abolished FoxD5b-En-mediated reporter activation which was instead observed with the wild-type reporter gene construct of *zic3*(-517)(Fig. 4B). These results indicate that FoxD5b-En-mediated promoter activation of *zic3*(-517) is at least partially dependent on the repression of endogenous Ventx1.1. Presently, we do not know whether FoxD5b-En enhances *zic3* expression through this negative regulation of *ventx1.1* alone or through a more complicated route, directly and/or indirectly affecting the *ventx1.1* action.

Xcad2 is one of the positive regulators of BMP4 signaling and is a homeobox transcriptional activator which induces the expression of *bmp4* and *ventx1.2* (Levy et al., 2002; Pillemer et al., 1998). The relative promoter activities of *zic3*(-517) were reduced in presence of Xcad2 (Fig. 4C, 1st and 2nd bar), and we asked whether Xcad2-mediated reduction in the relative promoter activity of *zic3*(-517) may also at least partially be regulated through the Ventx1.1 response element (VRE1) in *zic3* promoter. Interestingly, the site-directed mutagenesis construct of *zic3*(-517)mVRE1 abolished the Xcad2-mediated reduction in the relative promoter activity of wild-type construct of *zic3*(-517) (Fig. 4C, 3rd and 4th bar) and Xcad2 actually enhanced the relative promoter activity of *zic3*(-517)mVRE1. The Xcad2-mediated reduction in the relative promoter activity of *zic3*(-517) promoter construct was significantly increased compared with *zic3*(-517)mVRE1 promoter construct (Fig. 4C). Ventx1.1 also significantly increased the relative promoter activity of VRE1-mutated *zic3* promoter construct (Figs. 3D, 3E and 3I).

We observe that VRE1-mutated *zic3*(-517)mVRE1 promoter construct contains at least one negative cis-acting response element(s) where a putative transcription factor, which is decreased directly or indirectly by Xcad2, may bind. We suspect that *gsc* may be the factor which is decreased by ectopic expression of Xcad2 and negatively regulates the *zic3* promoter activity (based on ChIP-Seq data and reporter gene assays of *zic3*(-517), unpublished data). The identity of the factor has yet to be discovered. The results collectively indicate that both FoxD5b and Xcad2 positively and negatively regulate the reporter activity of *zic3*(-517), respectively, and in a VRE1-dependent manner. Based on these results, we propose that Ventx1.1 is a key molecule in the positive and negative circuits for ectoderm and neuro-ectoderm specification as well as ventral-dorsal specification of early *Xenopus* embryos.

Reciprocal repression on a preoccupied promoter region such as for determination of ectoderm vs. neuro-ectoderm and ventral vs. dorsal mesoderm is one of key mechanisms of germ layer specification

The first main event in neural tissue formation including for the central and peripheral nerve system is neuro-ectoderm formation from the naive ectoderm. In the ectoderm, high BMP4/Smad1 signal gradient induces the expression of the immediate early transcription factors (MZF genes), including *ventx1.1*. In this work, we demonstrated that one of the immediate early transcription factors induced by BMP4/Smad1

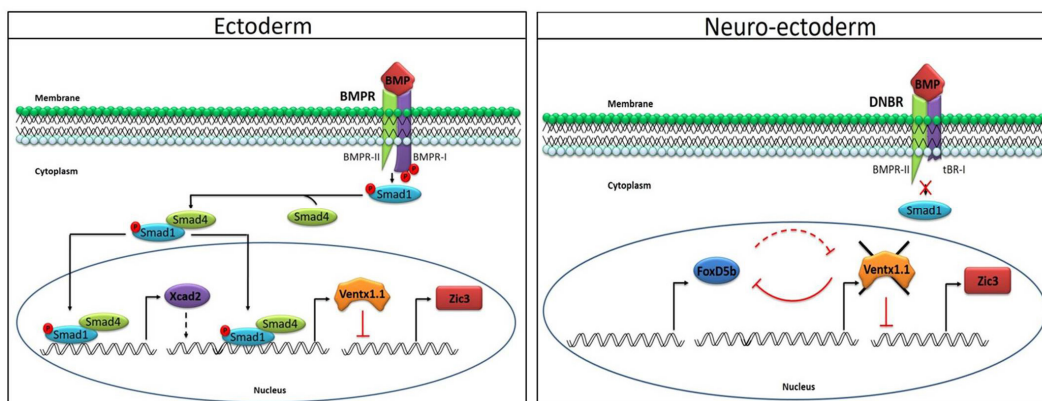


Fig. 5. Schematic diagram depicting Ventx1.1 as a key protein in the neural inhibitory circuit of BMP4/Smad1/Xcad2/Ventx1.1 and the reciprocal inhibitory circuit of FoxD5b/Ventx1.1. Ventx1.1 seems play an essential role in the BMP4/Smad1/Xcad2/Ventx1.1-mediated neural inhibition in *Xenopus* embryos during gastrula.

signal in the ectoderm directly represses the transcription of *zic3*, which is a commitment gene of the neuro-ectoderm. A genome-wide ChIP-Seq of Ventx1.1 showed that Ventx1.1 directly binds to the promoter regions of neural as well as organizer specific genes including *zic3*, *noggin*, *chordin*, *foxD5b* and *gsc* in *Xenopus* embryos (unpublished data). BMP inhibition by secreting organizer proteins also leads to expression of *zic3* and *foxD5b* in the neuro-ectoderm. In genome-wide ChIP-Seq for FoxD5b, FoxD5b directly binds to the promoter regions of *ventx1.1* to repress *ventx1.1* expression (unpublished data). Therefore, we hypothesize that a reciprocal repression networks exists among the germ layer specific repressor factors and suggest *ventx1.1* as a reciprocal repressor in the ectoderm and FoxD5b as a reciprocal repressor in the neuro-ectoderm environments. For the ectoderm vs. neuro-ectoderm determination, as a newly transcribed repressor, Ventx1.1 opposes *foxD5b* presence in the ectoderm while FoxD5b opposes *ventx1.1* presence in the neuro-ectoderm in order to repress the other factor's effect at a defined territory. In this study, we found that Ventx1.1 preoccupied the ectoderm region to repress the early neuro-ectoderm gene, *zic3*. Initially, BMP4 signaling is high at the ventral side and after the mid-blastula or mid-blastula transition (MBT), the immediate early target gene *ventx1.1* preoccupies this region and starts to repress the dorsal and neural specific genes in order to maintain the epidermal fate and its differentiation. Xcad2 as one of the downstream target genes of the BMP4/Smad1 pathway is expressed around the blastopore and positively contributes to the expression of *ventx1.1* for indirect repression of *zic3* expression (Fig. 5. left panel). FoxD5b, on the other hand, is expressed as a neural specific repressor in the neuro-ectodermal region. FoxD5b at least partially contributes to the expression of another neural gene, namely *zic3*, through repression of *ventx1.1* (Fig. 5. right panel). Based on the results for our reciprocal repression model, a putative mechanism of *zic3* transcriptional regulation in ectoderm and neuro-ectoderm regions during gastrula of *Xenopus* embryos is proposed (Fig. 5).

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