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**Ventx1.1 competes with a transcriptional activator Xcad2 to regulate negatively its own expression**

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**ABSTRACT**

Dorsoventral patterning of body axis in vertebrate embryo is tightly controlled by a complex regulatory network of transcription factors. *Ventx1.1* is known as a transcriptional repressor to inhibit dorsal mesoderm formation and neural differentiation in *Xenopus*. In an attempt to identify, using chromatin immunoprecipitation (ChIP)-Seq, genome-wide binding pattern of *Ventx1.1* in *Xenopus* gastrulae, we observed that *Ventx1.1* associates with its own 5'-flanking sequence. In this study, we present evidence that *Ventx1.1* binds a *cis*-acting *Ventx1.1* response element (VRE) in its own promoter, leading to repression of its own transcription. Site-directed mutagenesis of the VRE in the *Ventx1.1* promoter significantly abrogated this inhibitory autoregulation of *Ventx1.1* transcription. Notably, *Ventx1.1* and *Xcad2*, an activator of *Ventx1.1* transcription, competitively co-occupied the VRE in the *Ventx1.1* promoter. In support of this, mutation of the VRE down-regulated basal and *Xcad2*-induced levels of *Ventx1.1* promoter activity. In addition, overexpression of *Ventx1.1* prevented *Xcad2* from binding to the *Ventx1.1* promoter, and vice versa. Taken together, these results suggest that *Ventx1.1* negatively regulates its own transcription in competition with *Xcad2*, thereby fine-tuning its own expression levels during dorsoventral patterning of *Xenopus* early embryo.

**Keywords:** *Ventx1.1*, Transcription, *Xcad2*, Autoregulation, *Xenopus*

## INTRODUCTION

Bone morphogenetic protein 4 (BMP4) is a member of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily of secreted signaling proteins. It controls via Smad1/5/8 the transcription of tissue-specific target genes during early development and adult tissue homeostasis (1). *Ventx1.1* (PV.1, Xvent-1b) and *Xcad2*, homeobox transcription factors, are downstream target genes of BMP4/Smad1 signaling in *Xenopus* embryos (2, 3). *Ventx1.1* acts as a transcriptional repressor to inhibit the expression of neural and organizer-specific genes, including *FoxD5b*, *Zic3*, *NCAM*, *Otx2*, *Gsc*, *Noggin*, and *Chordin* (2, 4-6). Thus, the ectopic expression of *Ventx1.1* causes ventralized and headless embryonic phenotypes. *Xcad2* is a transcriptional activator that can induce the expression of *BMP4* as well as of its downstream target genes such as *Ventx1.1*, *Ventx1.2*, *Ventx2.1*, and *Xpo* (3). However, it inhibits the expression of organizer-specific genes *Gsc*, *Xlim1*, and *Otx2* independently of BMP signaling (3). Recently, it has been shown that the BMP4/Smad1 pathway can synergize with the FGF/Xbra pathway to activate *Ventx1.1* transcription, resulting in neural inhibition in *Xenopus* embryos (7, 8). In line with this, 5'-flanking upstream region of *Ventx1.1* contains direct *cis*-acting responsive elements for several transcription factors, including Smad1, Xbra, and OAZ. While the repressive effects of *Ventx1.1* on the expression of various neural and organizer genes have been demonstrated, its global binding sites remain to be characterized. Thus, we sought to identify genome-wide occupancy pattern of *Ventx1.1* in *Xenopus* gastrulae using chromatin immunoprecipitation (ChIP)-Seq. As expected, our analysis revealed that neural and organizer-specific genes were included in the list of candidates whose 5'-flanking region might contain binding sites for *Ventx1.1*. Notably, *Ventx1.1* could also bind to its own 5'-flanking upstream sequence.

In this study, we have identified direct response element for Ventx1.1 to down-regulate its own transcription in a negative feedback loop. Intriguingly, Ventx1.1 and Xcad2, an activator of *Ventx1.1* transcription, competitively co-occupied the common binding site within the 5'-promoter region of *Ventx1.1*. These results provide an insight into the mechanism by which the expression level of *Ventx1.1* is fine-tuned spatially and temporally during dorsoventral patterning of *Xenopus* embryo.

## RESULTS AND DISCUSSION

### **Overexpression of Ventx1.1 inhibits the expression of ventral-specific genes**

Our ChIP-Seq analysis showed that Ventx1.1 might directly bind to not only its own 5'-flanking sequence but that of *Ventx1.2* or *Ventx2.1* in *Xenopus* gastrula-stage embryos (data not shown). Thus, we first examined the effects of the gain-of-Ventx1.1 function on the expression of ventral-specific genes. As shown in Fig. 1A, B, overexpression of *Ventx1.1* reduced its own endogenous expression and that of other ventral genes such as *Ventx1.2* and *Ventx2.1* in animal caps as analyzed by RT-PCR. In contrast, Ventx1.1 had no effect on the expression of *BMP4*. Given that these ventral genes are BMP4-induced targets (9), these results suggest that Ventx1.1 can down-regulate the expression of ventral-specific genes, including itself, in a BMP-independent manner.

### **Mapping of Ventx1.1 response element in its own promoter**

Negative autoregulation of transcription of a gene can restrict its expression level and/or activity, contributing to proper patterning of embryonic body axis (10, 11). Of potential candidate genes whose 5'-promoter region might contain Ventx1.1-responsive elements, we

chose *Ventx1.1* itself for this study to gain further mechanistic insight into the regulation of its expression. Analysis of the 5'-flanking genomic sequence (2.5 kb) of *Ventx1.1* revealed 13 putative cis-acting response elements for *Ventx1.1* (Supplementary Table 1). To identify functional *Ventx1.1* response elements (VREs) within the flanking region, we generated luciferase reporter constructs containing serially-deleted *Ventx1.1* promoter fragments (Fig. 2A) and then examined the effects of overexpression of *Ventx1.1* on their promoter activities. Co-expression of *Ventx1.1* significantly reduced the activity of the -2525-bp construct (Fig. 2B), indicating the presence of functional VREs in the full 5'-flanking sequence. Of note, the inhibitory effect of *Ventx1.1* on the reporter activity became relatively stronger as gastrulation proceeded (0.7-fold at stage 11.5 as compared with 0.5-fold at stage 10.5). While *Ventx1.1*-mediated repression of the reporter activity was still observed when the promoter fragment was reduced to -951 bp, this effect was lost upon deletion to -399 bp (Fig. 2C). This suggests that *Ventx1.1* response elements are located between -951 bp and -399 bp. The promoter region between -951 bp and -399 bp contains two putative *Ventx1.1* binding sites [VRE1, -626 bp to -621 bp (GATTTG) and VRE2, -697 bp to -692 bp (GATTTT)] (Supplementary Table 1). To determine whether these two potential sites could confer *Ventx1.1* responsiveness, we substituted 5'-GGGG-3' for the core sequence 5'-ATTT-3' in the putative VRE1 or VRE2 in the -951-bp promoter construct and measured activities of these mutated reporters in the absence or presence of *Ventx1.1*. Interestingly, mutation of VRE1 (-951 bp-mVRE1), but not of VRE2 (-951 bp-mVRE2), resulted in a significant decrease (13-fold) in the basal promoter activity of the -951-bp construct even in the absence of *Ventx1.1* (Fig. 2D, E), suggesting that the putative VRE1 might also act as a binding site for a transcriptional activator that could positively regulate

*Ventx1.1* expression. Furthermore, unlike the wild-type -951-bp reporter construct, the -951-bp-mVRE1 reporter was unresponsive to co-injected *Ventx1.1* (Fig. 2E), supporting the functionality of VRE1. In contrast, the -951-bp-mVRE2 promoter fragment still exhibited significantly reduced activity upon co-expression of *Ventx1.1* (Fig. 2E), indicating that the putative VRE2 might not be relevant to inhibitory autoregulation of *Ventx1.1* expression. In addition, we performed ChIP-PCR analysis to test whether *Ventx1.1* might indeed bind to the promoter region encompassing VRE1. As shown in Fig. 2F, anti-Flag antibody-mediated ChIP followed by PCR showed that Flag-*Ventx1.1* was highly enriched in the promoter fragment containing VRE1. Taken together, these results suggest that *Ventx1.1* acts as a repressor of its own transcription by binding to the response element present in its own promoter.

### **Xcad2 activates *Ventx1.1* transcription by binding to VRE1**

As shown above, the -951-bp promoter with VRE1 mutation displayed significantly reduced basal activity, suggesting that the response element might be co-occupied by a transcriptional activator as well as *Ventx1.1* itself as a repressor. It is known that the expression pattern of *Xcad2* overlaps with that of *Xvent-1* in *Xenopus* gastrulae and that *Xcad2* can positively regulate the expression of ventral-specific genes including *Ventx1* and *Ventx2* (3, 12). Thus, *Xcad2* might be a potential candidate capable of binding to VRE1 to activate *Ventx1.1* transcription. As expected, forced expression of *Xcad2* increased the transcription of ventral genes such as *Ventx1.1*, *Ventx1.2* and *Ventx2.1* and *BMP4*, in animal caps as analyzed by RT-PCR (Fig. 3A). Conversely, overexpression of *Ventx1.1* down-regulated basal and *Xcad2*-induced levels of expression of ventral genes except

*BMP4*. These results suggest that *Xcad2* can act as a transcriptional activator of ventral genes to counteract inhibitory effects of *Ventx1.1*.

Response elements for caudal-type transcription factors such as *Xcad2* have a core consensus sequence, 5'-ATTT-3' (13-15). Note that VRE1 in the promoter region of *Ventx1.1* also contains this core sequence (Supplementary Table 1). Consistent with this, overexpression of *Xcad2* increased the activity of the -951-bp construct to ~3-fold over the basal level, but had no effect on the activity of its version with VRE1 mutation (Fig. 3B). These results indicate that *Xcad2* could up-regulate *Ventx1.1* expression by occupying VRE1 within its promoter region. Thus, we next examined whether *Xcad2* might indeed bind to VRE1 to activate *Ventx1.1* transcription. As shown in Fig. 3C, overexpressed *Xcad2* associated with VRE1 in the promoter region of *Ventx1.1* as analyzed by ChIP-PCR. In addition, co-expression of *Ventx1.1* caused displacement of *Xcad2* from the DNA binding site (Fig. 3C, D), suggesting that VRE1 might be shared by both of the transcription factors. In line with this, overexpression of *Xcad2* induced an increase in the activity of the -951-bp promoter fragment, which could be abrogated by co-expression of *Ventx1.1* (Fig. 3E). Taken together, these results indicate that *Xcad2* binds to VRE1 to up-regulate *Ventx1.1* transcription.

#### **Ventx1.1 and Xcad2 competitively occupy VRE1 to regulate Ventx1.1 transcription**

Co-occupancy of a promoter region by two transcription factors suggests their competition in control of gene expression. Thus, we further investigated whether *Ventx1.1* and *Xcad2* could interfere with each other in binding to VRE1. As demonstrated by ChIP-PCR experiments, overexpressed *Ventx1.1* could bind to its own promoter containing VRE1,

while such occupancy by Ventx1.1 was gradually decreased by increasing co-expression of *Xcad2* (Fig. 4A, B). In addition, the association between *Xcad2* and *Ventx1.1* promoter was markedly inhibited by co-expression of *Ventx1.1* (Fig. 4C, D). These results suggest that *Xcad2* and Ventx1.1 can competitively occupy the *cis*-acting response element within the promoter region of *Ventx1.1* to regulate its transcription in an opposite way.

Ventx1.1 functions as a transcriptional repressor to exert anti-organizer and anti-neural activities, mimicking ventralizing effects of BMP4/Smad1 signaling (2, 4). *Ventx1.1* is a direct target of the BMP4/Smad1 pathway. It displays an expression pattern reminiscent of that of *BMP4* along the marginal and animal regions of early gastrulae. Expectedly, the *cis*-acting response elements for Smad1 and a cofactor OAZ as well as positive regulatory factors Xvent-2 and GATA2 have been identified in the promoter region of *Ventx1.1* (8, 16). In this study, we also identified *Xcad2* as an activator of *Ventx1.1* transcription that directly occupied a response element in its promoter sequence (Fig. 4E), which was distinct from that for Smad1, OAZ, or Xvent-2. Mutation of this response element significantly abrogated basal and *Xcad2*-induced activation of the -951-bp *Ventx1.1* promoter, which still contained intact response elements for Smad1, OAZ, and Xvent-2. As such, the contribution of the *Xcad2*-responsive element to peak activation of the *Ventx1.1* promoter would be highly significant. Our previous study has revealed that *Xbra*, a target of FGF signaling, also cooperates with Smad1 to up-regulate the activity of the *Ventx1.1* promoter in a synergistic manner (7). Expression of *Xcad2* and *Xvent-2* can be induced by not only BMP signal, but also by combined activity of Wnt and FGF signaling pathways (3, 12, 17, 18). Thus, it appears that transcriptional cofactors *Xbra*, *Xcad2*, and *Xvent-2* can act as mediators of cross-talk between BMP and other signals such as Wnt and FGF in the control

of *Ventx1.1* transcription. Given that expression patterns of *Ventx1.1* and its upstream regulators *Xbra*, *Xcad2*, and *Xvent-2* in *Xenopus* gastrulae are overlapped (3), these transcriptional cofactors appear to play roles in restricting *Ventx1.1* expression both spatially and temporally. On the other hand, *Xbra*, *Xcad2*, or *Xvent-2* was unable to efficiently induce *Ventx1.1* expression in the absence of BMP signaling (3, 9, 16), indicating the necessity of their cooperation with basic regulatory factors activated by BMP signal. Taken together, these results suggest that these transcriptional factors primarily contribute to maximal activation rather than basal activity of *Ventx1.1* transcription.

It has been shown that *Gooseoid* acts as a repressor of *Ventx1.1* transcription to oppose its ventralizing activity (19). Consistently, a response element for *Gooseoid* is present in the *Ventx1.1* promoter, and overexpression of *Gooseoid* suppresses its activity (8). Since *Gooseoid* is expressed on the dorsal side of *Xenopus* early gastrulae, the absence of *Ventx1.1* transcription in that region might be primarily due to *Gooseoid*-mediated repression of its promoter activity. Notably, we observed that *Ventx1.1* down-regulated its own transcription by binding to a response element within its own promoter (Fig. 4E). An intriguing aspect of the *Ventx1.1* response element in its own promoter is that this sequence is competitively co-occupied by *Ventx1.1* and a transcriptional activator *Xcad2*. This feature suggests that negative auto-regulation of *Ventx1.1* transcription occurs in tissues where *Ventx1.1* is endogenously expressed such as the ventrolateral marginal region of *Xenopus* gastrulae. In addition, since *Xcad2* is required for the peak activity of *Ventx1.1* promoter, it is reasonable to speculate that the inhibitory auto-regulation of *Ventx1.1* transcription might be a key regulatory mechanism to maintain *Ventx1.1* expression at a moderate level. Overexpression of *Ventx1.1* abolished the expression of endogenous

*Ventx1.1* as well as of its upstream regulator *Xvent-2* (Fig. 1), possibly resulting in significant decrease of *Ventx1.1* expression below moderate level. As shown in our CHIP-PCR assays (Fig. 4), *Ventx1.1* appeared to displace *Xcad2* more easily from the co-occupied response element than *Xcad2*, suggesting a stronger association of *Ventx1.1* with the promoter. This strong binding affinity of *Ventx1.1* might ensure immediate control of its own expression in the presence of transcriptional activators such as *Xcad2*. In this respect, fine-tuning of relative cellular levels of *Ventx1.1* and *Xcad2* might play critical roles in the establishment of optimal level of *Ventx1.1* expression, contributing to proper dorsoventral patterning of early embryo. Of note, it has also been shown that *Gooseoid* can negatively control its own promoter activity for balanced organizer activity (11). Thus, *Ventx1.1* and *Gooseoid* appear to repress each other, leading to their separate expression on the ventral and dorsal sides of embryo, and negatively regulate their own transcription to achieve appropriate levels of their respective expression.

## MATERIALS AND METHODS

### Embryo manipulation

*Xenopus laevis* were obtained from the Korean *Xenopus* Resource Center for Research. Embryos were obtained by *in vitro* fertilization after induction of female frogs with 500 units of human chorionic gonadotropin (Sigma). mRNAs and/or DNA constructs were injected into the animal pole region of one-cell stage embryos. For animal cap assays, animal cap explants were dissected from injected or uninjected embryos at stage 8 and incubated in L-15 medium to the desired stages for subsequent experiments.

### **Ventx1.1 (Xvent-1b, PV.1) genomic DNA and promoter constructs**

Genomic library screening for the isolation of *Ventx1.1a* genomic DNA was previously described (8). A 3.8-kb DNA fragment from the library screening, which contained 2.5 kb of 5'-flanking region of *Ventx1.1*, was subcloned into pGL-2 basic plasmid (Promega) to produce a -2525-bp promoter construct. Serially-deleted *Ventx1.1* promoter constructs were generated by subcloning its promoter fragments, which were obtained from the -2525-bp construct by PCR amplification (Supplementary Table 2), into a pGL-2 basic plasmid.

### **Site-directed mutagenesis**

Mutagenesis was performed using Muta-Direct™ site-directed mutagenesis kit (iNiRON) in accordance with manufacturer's instructions. Primers used for mutagenesis of VRE1 and VRE2 were as follows: VRE1, (forward) 5'-CATCCTGCTGGCGGGGGTTCATTCTAGCTG-3' and (reverse) 5'-CAGCTAGAATGAACCCCGCCAGCAGGATG-3'; VRE2, (forward) 5'-CAAAG AAGAGGAGGGGGTTCGCTGGGGCAA-3' and (reverse) 5'-TTGCCCCAGCGACCCCTC CTCTTCTTTG-3'.

### **RNA synthesis**

Capped synthetic mRNAs were *in vitro* generated using a MEGAscript kit (Ambion) according to manufacturer's instructions. Expression constructs *Flag-Ventx1.1* and *Myc-Xcad2* were linearized with *SacII* and *Asp718*, respectively, and their respective mRNAs were synthesized using SP6 RNA polymerase.

### **Chromatin Immunoprecipitation (ChIP) assay**

For ChIP assay, embryos were injected at one-cell stage with *Flag-Ventx1.1* (0.5 ng/embryo) and *Myc-Xcad2* (1 ng/embryo) mRNA either alone or together, collected at stage 11 (100 embryos/sample) and processed according to published protocol (20). Anti-Flag (Sigma, F-1804) monoclonal antibody and anti-Myc (Santa Cruz, SC-789) polyclonal antibody were used to immunoprecipitate chromatin fragments. Normal rabbit IgG (Santa Cruz, SC-2027) and mouse IgG (Santa Cruz, SC-2025) were used as negative controls. Primers used for PCRs are listed in Supplementary Table 2 and 3.

### **RNA isolation and RT-PCR**

Total RNA was isolated from whole embryos or animal caps using RNA-Bee reagent (TEL-TEST) and treated with DNase I to remove genomic DNA contamination. cDNAs were synthesized using SuperScript IV (Invitrogen) with 2 µg total RNA per reaction. PCRs were performed as follows: 30 seconds at 94°C, 30 seconds at 57°C and 30 seconds at 72°C; 20-26 cycles of amplification (Supplementary Table 3).

### **Western blotting**

Whole embryos were homogenized in lysis buffer containing phosphatase and protease inhibitors. Proteins were resolved on 12% SDS-PAGE. The following antibodies were used: anti-Flag monoclonal (Sigma, F-1804) and anti-Myc polyclonal (Santa Cruz, SC-805) primary antibodies, and anti-rabbit IgG (Santa Cruz, SC-789) and anti-mouse IgG (Stressgen, SAB-100) secondary antibodies. Protein bands were visualized using an ECL detection kit (GE healthcare).

**Luciferase reporter assay**

Luciferase reporter assays were carried out using luciferase assay system (Promega) according to the manufacturer's instructions. Five different groups of embryos (3 embryos/group) were harvested and homogenized in lysis buffer (10  $\mu$ l/embryo). Embryo lysates (10  $\mu$ l) were assayed with luciferase substrate (40  $\mu$ l), and relative luciferase activity was determined by luminometer (EG &G, Berthold). Independent experiments were repeated at least three times.

**Statistical analysis**

All data were analyzed using GraphPad Prism4. Statistical analysis was performed with one-way analysis of variance (ANOVA). A  $p$  value of less than 0.05 was considered statistically significant (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s., not significant).

**Nucleotide sequence accession number**

*Ventx1.1* cDNA sequence was deposited at GenBank (accession number: AF133122).

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**CONFLICTS OF INTEREST**

The authors have no conflicting interests.

**FIGURE LEGENDS**

**Fig. 1.** *Ventx1.1* represses expression of ventral-specific genes. (A) One-cell stage embryos were injected in the animal pole region with *Flag-Ventx1.1* mRNA (500 pg), and animal caps were dissected from injected or uninjected embryos at stage 8 and cultured to stage 11.5 for RT-PCR analysis. *EF1 $\alpha$*  serves as a loading control. WE, stage 11.5 whole embryo. AC, animal cap. -RT, control in the absence of reverse transcriptase. (B) Quantification of expression levels of ventral genes (normalized to *EF1 $\alpha$* ) from three independent experiments for (A). Error bars indicates standard error (SE).

**Fig. 2.** Identification of a *Ventx1.1*-responsive element (VRE) in its own promoter region. (A) Diagram of *Ventx1.1* promoter deletion constructs. The length that each promoter fragment extends upstream of the major *Ventx1.1* transcription initiation site is indicated at left. Each promoter fragment was fused to luciferase (LUC) reporter gene. (B-E) Embryos were injected at one-cell stage with wild-type or putative VRE-mutated *Ventx1.1* promoter deletion constructs alone or with *Flag-Ventx1.1* mRNA (500 pg) as indicated, cultured to stage 10.5 (B) or 11.5 (B-E), and harvested for luciferase reporter assays. All relative promoter activity data are shown as mean  $\pm$  standard error (SE). (F) ChIP-PCR analysis showing the occupancy by *Ventx1.1* of its own promoter region. *Ventx1.2*, a negative control for PCR, was amplified using its coding region-specific primers. IgG, a negative control IgG.

**Fig. 3.** *Xcad2* up-regulates *Ventx1.1* transcription by occupying VRE1. (A) Animal caps from embryos injected or not with *Flag-Ventx1.1* (500 pg) and/or *Myc-Xcad2* (1 ng)

mRNA as indicated were subjected to RT-PCR analysis at stage 11.5. (B-E) One-cell stage embryos were injected with indicated combinations of -951-bp reporter, -951-bp-mVRE1 reporter, *Flag-Ventx1.1* (500 pg), and *Myc-Xcad2* (1 ng) and cultured to stage 11 for ChIP-PCR (C and D) or stage 11.5 for luciferase reporter assay (B and E). (D) Quantification of relative intensities of bands in lanes 2, 5, and 6 shown in (C).

**Fig. 4.** *Ventx1.1* and *Xcad2* co-occupy *Ventx1.1* promoter region in competition with each other. (A-D) Embryos were injected with a combination of *Flag-Ventx1.1* (0.5 ng) and increasing doses of *Myc-Xcad2* (0.5, 1 ng) or *Myc-Xcad2* (1 ng) and increasing doses of *Flag-Ventx1.1* (0.25, 0.5 ng) as indicated and cultured to stage 11.5 for ChIP-PCR analysis. (B) and (D) represent quantification of relative intensities of bands in lanes 1, 2, and 3 in (A) and (C), respectively. (-), no injection of *Myc-Xcad2* or *Flag-Ventx1.1*. *Ventx2.1*, a negative PCR control, was amplified with its coding region-specific primers. (E) A proposed model for *Ventx1.1* or *Xcad2*-mediated control of *Ventx1.1* transcription.

## REFERENCES

1. Massague J (2008) TGFbeta in Cancer. *Cell* 134, 215-230
2. Ault KT, Dirksen ML and Jamrich M (1996) A novel homeobox gene PV.1 mediates induction of ventral mesoderm in *Xenopus* embryos. *Proc Natl Acad Sci U S A* 93, 6415-6420
3. Pillemer G, Yelin R, Epstein M et al (1998) The Xcad-2 gene can provide a ventral signal independent of BMP-4. *Mech Dev* 74, 133-143
4. Ault KT, Xu RH, Kung HF and Jamrich M (1997) The homeobox gene PV.1 mediates specification of the prospective neural ectoderm in *Xenopus* embryos. *Dev Biol* 192, 162-171
5. Hwang YS, Lee HS, Roh DH et al (2003) Active repression of organizer genes by C-terminal domain of PV.1. *Biochem Biophys Res Commun* 308, 79-86
6. Hwang YS, Seo JJ, Cha SW et al (2002) Antimorphic PV.1 causes secondary axis by inducing ectopic organizer. *Biochem Biophys Res Commun* 292, 1081-1086
7. Kumar S, Umair Z, Yoon J et al (2018) Xbra and Smad-1 cooperate to activate the transcription of neural repressor ventx1.1 in *Xenopus* embryos. *Sci Rep* 8, 11391
8. Lee HS, Lee SY, Lee H et al (2011) Direct response elements of BMP within the PV.1A promoter are essential for its transcriptional regulation during early *Xenopus* development. *PLoS One* 6, e22621
9. Yoon J, Kim JH, Lee SY et al (2014) PV.1 induced by FGF-Xbra functions as a repressor of neurogenesis in *Xenopus* embryos. *BMB Rep* 47, 673-678

10. Charney RM, Paraiso KD, Blitz IL and Cho K W Y (2017) A gene regulatory program controlling early *Xenopus* mesendoderm formation: Network conservation and motifs. *Semin Cell Dev Biol* 66, 12-24
11. Danilov V, Blum M, Schweickert A, Campione M and Steinbeisser H (1998) Negative autoregulation of the organizer-specific homeobox gene *gooseoid*. *J Biol Chem* 273, 627-635
12. Keenan ID, Sharrard RM and Isaacs HV (2006) FGF signal transduction and the regulation of *Cdx* gene expression. *Dev Biol* 299, 478-488
13. Ryu JH, Nam KB, Oh CT et al (2004) The homeobox gene *Caudal* regulates constitutive local expression of antimicrobial peptide genes in *Drosophila* epithelia. *Mol Cell Biol* 24, 172-185
14. Hwang MS, Kim YS, Choi NH et al (2002) The caudal homeodomain protein activates *Drosophila* *E2F* gene expression. *Nucleic Acids Res* 30, 5029-5035
15. Suh E, Chen L, Taylor J and Traber PG (1994) A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol Cell Biol* 14, 7340-7351
16. Friedle H and Knochel W (2002) Cooperative interaction of *Xvent-2* and *GATA-2* in the activation of the ventral homeobox gene *Xvent-1B*. *J Biol Chem* 277, 23872-23881
17. Onichtchouk D, Gawantka V, Dosch R et al (1996) The *Xvent-2* homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controlling] dorsoventral patterning of *Xenopus* mesoderm. *Development* 122, 3045-3053

18. Hikasa H, Ezan J, Itoh K, Li X, Klymkowsky MW and Sokol SY (2010) Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. *Dev Cell* 19, 521-532
19. Sander V, Reversade B and De Robertis EM (2007) The opposing homeobox genes Goosecoid and Vent1/2 self-regulate *Xenopus* patterning. *EMBO J* 26, 2955-2965
20. Blythe SA, Reid CD, Kessler DS and Klein PS (2009) Chromatin immunoprecipitation in early *Xenopus laevis* embryos. *Dev Dyn* 238, 1422-1432

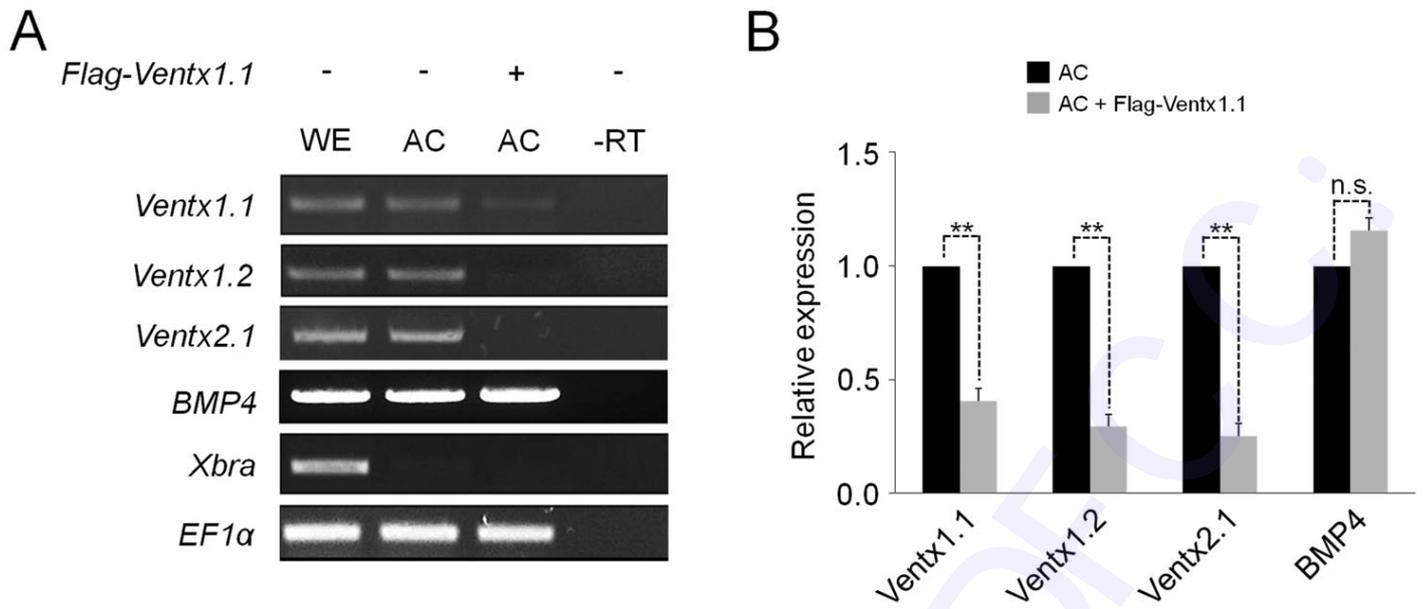


Fig. 1. Figure 1

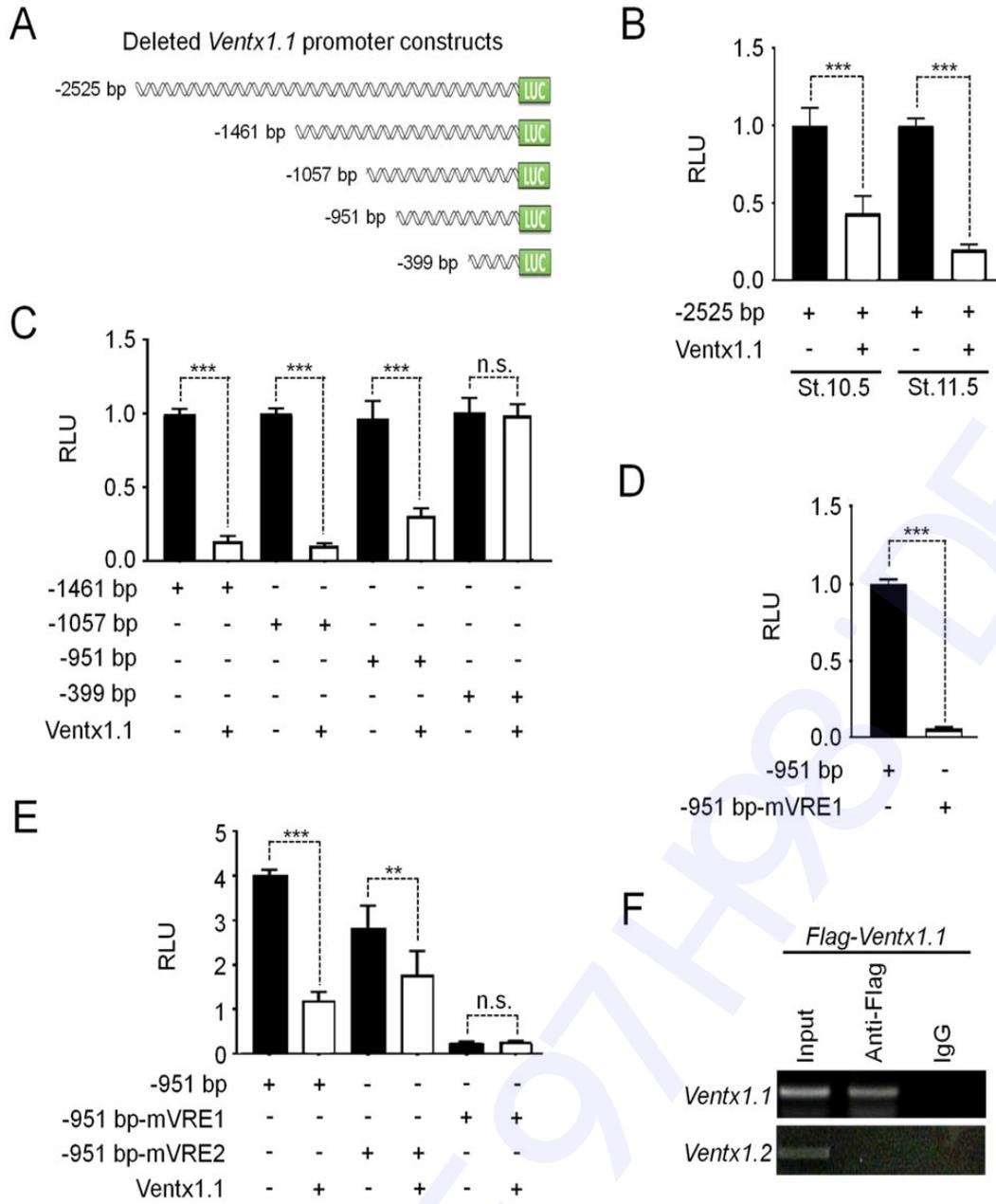


Fig. 2. Figure 2

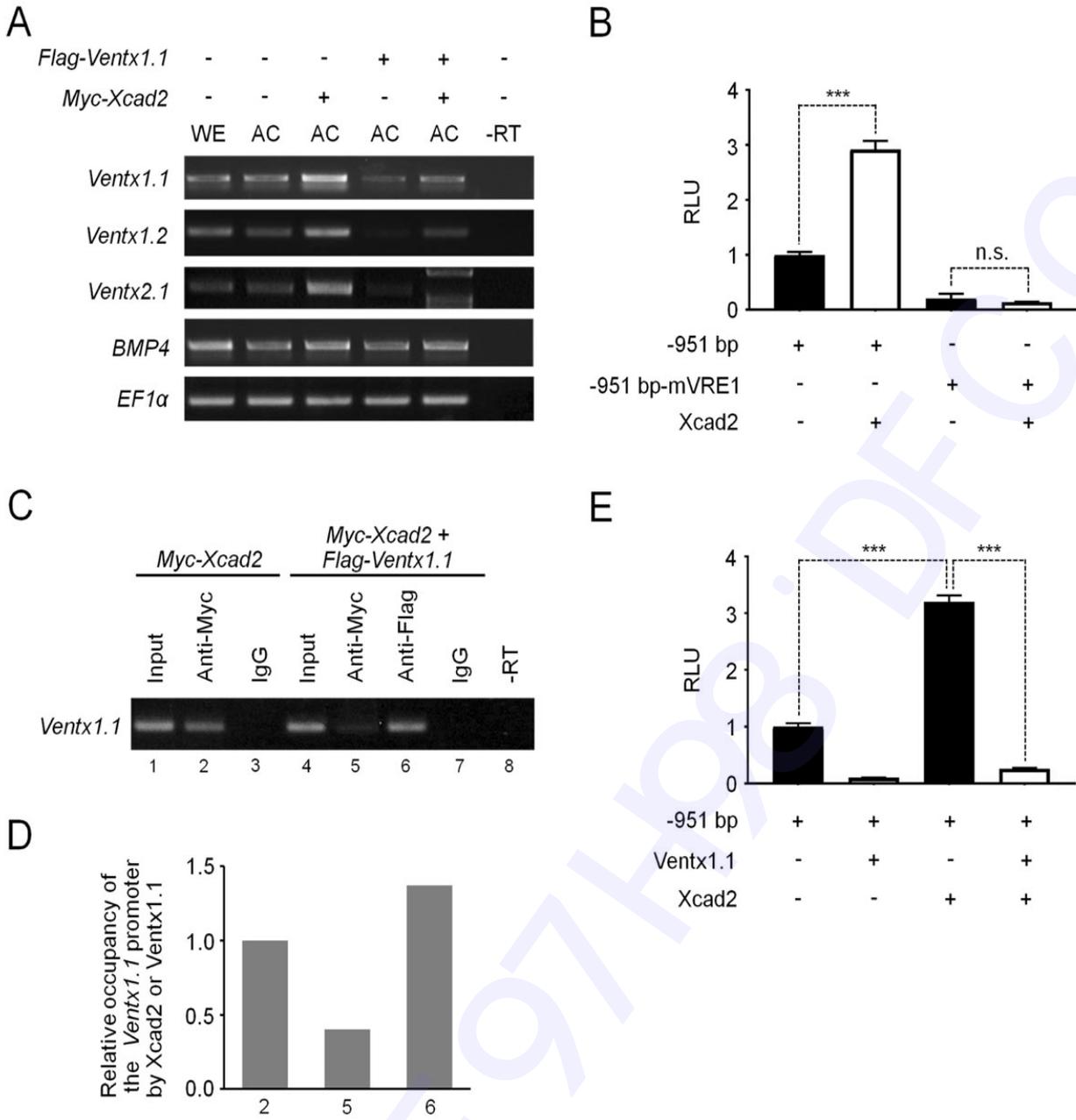


Fig. 3. Figure 3

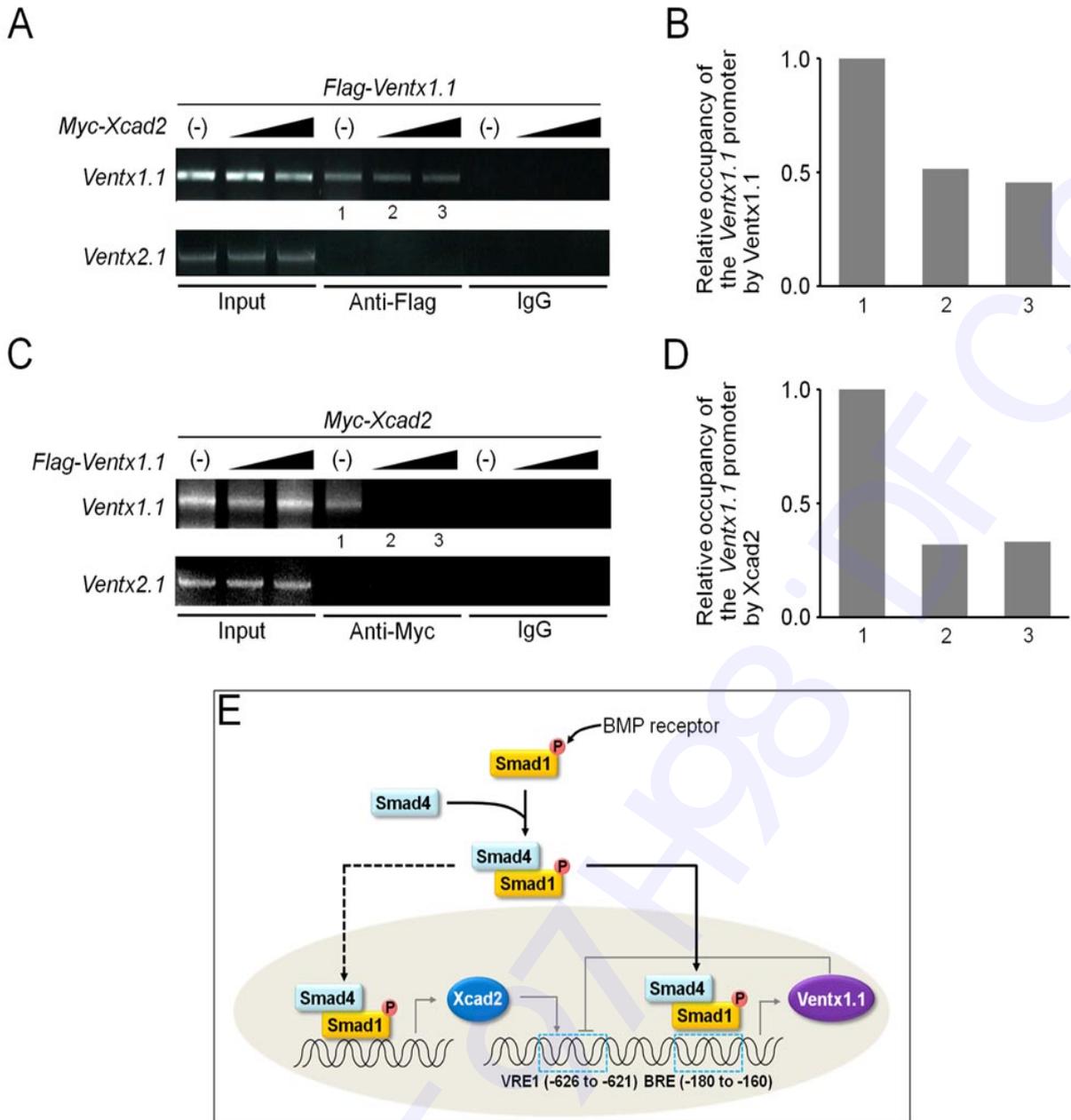


Fig. 4. Figure 4

**Supplementary Table 1. Putative binding sites for Ventx1.1 in the promoter region of *Ventx1.1***

Location of putative binding sites in 5'-flanking sequence of <i>Ventx1.1</i>	Putative binding sequence	Location of putative binding sites in 5'-flanking sequence of <i>Ventx1.1</i>	Putative binding sequence
-2075 bp to -2070 bp	TATTTT	-1506 bp to -1501 bp	GATTTT
-1990 bp to -1995 bp	TATTTC	-1346 bp to -1341 bp	TATTTT
-1952 bp to -1947 bp	TATTTC	-1266 bp to -1261 bp	TATTTT
-1942 bp to -1937 bp	TATTTA	-1098 bp to -1093 bp	GATTTG
-1874 bp to -1869 bp	GATTTT	-697 bp to -692 bp	GATTTT
-1718 bp to -1713 bp	GATTTT	<b>-626 bp to -621 bp (VRE1)</b>	<b>GATTTG</b>
-1588 bp to -1583 bp	GATTTT		

**Supplementary Table 2. List of primers used to generate the serially-deleted reporter constructs**

	Primer name	Sequence (5' → 3')
Upstream Primers	-2525	AGTCCTCGAGTACCTGCAACTTACTCGC
	-1461	AGTCCTCGATAACCCCCTAAGTGTTTC
	-1057	AGTCCTCGAGCAGAACGTGTGGATAACAGGT
	-951	AGTCCTCGATAGCTTCTAGGTGTTAAAAGA
	-399	AGTCCTCGAGCCAGTCTCCTGGTGTGACTT
Downstream Primer		AGTCAAGCTTGATGGAGCCGCTGGAGTTGTG

**Supplementary Table 3. List of primers used for PCR amplification**

Gene name	Sequence	Annealing Temperature (°C)	Cycles
<i>BMP4</i>	F: 5'-GCATGTACGGATAAGTCGATC-3' R: 5'-GATCTCAGACTCAACGGCAC-3'	57	25
<i>Ventx1.1</i>	F: 5'-CCTTCAGCATGGTTCAACAG-3' R: 5'-CATCCTTCTTCCTTGGCATCTCCT-3'	57	26
<i>Ventx1.2</i>	F: 5'-TTCCCTTCAGCATGGTTCAAC-3' R: 5'-GCATCTCCTTGGCATATTTGG-3'	57	25
<i>Ventx2.1</i>	F: 5'-CTACAGCACTAGCACTGACTCAGG-3' R: 5'-TTGGACTGCATGCTGCAATACAGG-3'	57	25
<i>Xbra</i>	F: 5'-GGATCGTTATCACCTCTG-3' R: 5'-GTGTAGTCTGTAGCCAGCA-3'	57	25
<i>EF1<math>\alpha</math></i>	F: 5'-CCTGAATCACCCAGGCCAGATTGTG-3' R: 5'-GAGGGTACTCTGAGAAGCTCTCCACG-3'	57	20