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## Original Article

# Sox31 is involved in central nervous system anteroposterior regionalization through regulating the organizer activity in zebrafish

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**Sox superfamily proteins are DNA-binding transcriptional factors that contain highly conserved high-mobility group (HMG) box and take part in various development process. Sox31 is a maternal factor supplied in the oocyte and starts its zygotic expression during mid-blastula transition (MBT). From gastrulation stage, it mainly resides in neural tissue. Ectopically expression of *Sox31* mRNA leads to cyclopia, fusion eyes, or totally loss of anterior head structure, in accompany with severe notochord defects. Molecular markers indicate that forebrain tissue reduces sharply while the posterior neural tissue expands anteriorly. In addition, organizer specification is also suppressed. Oppositely, an antisense morpholino designed functionally knockdown *Sox31* causes typically dorsalized phenotype and reversed central nervous system (CNS) anteroposterior (AP) patterning. Gain of function with chimeric construct, where Sox31 HMG DNA binding domain is fused to a transcription activation domain (VP16) or transcription suppression domain (EnR), suggests that Sox31 acts as a transcriptional suppressor *in vivo*. The expression of *Bozozok* (*Dharma*), a direct target gene of pre-MBT Wnt/β-catenin signal, is suppressed by Sox31. Thus, to unveil the relationship between Sox31 and β-catenin-related transcriptional activity, we designed Top/Fop luciferase assay in HEK293T cells, and found that Sox31 could indeed suppress Tcf/Lef-dependent transcriptional activity without influencing the stability of β-catenin. Moreover, post-MBT Wnt signal was reduced in *Sox31* morphants corresponding to the suppressed hindbrain structure, while phenotypic defects caused by excessive Sox31 could be rescued by Wnt antagonist dkk1. Taken together, Sox31 functions as an essential CNS AP patterning determinant and coordinates the CNS AP patterning process with organizer specification.**

*Keywords*    Sox31; organizer specification; central nervous system AP patterning; mid-blastula transition

## Introduction

During vertebrate development, anteroposterior (AP) and dorsoventral axes are established by inductive signals that are secreted by discrete groups of cells known as Spemann organizer. When this structure is grafted to the ventral side or ventralized embryo, it can elicit secondary notochord, somites, and neural tissues [1]. Besides defining the future dorsal side of embryo, the organizer also plays a key role in neural induction and neural AP axis patterning following the Nieuwkoop's two-step model [2]. During mesendoderm involution process, the organizer secretes bone morphogenic protein (BMP) antagonist Noggin, Chordin, Follistatin, and Cerberus, which induce the overlying ectoderm toward neural fate. The newly induced neuroectoderm is characteristically anterior and subjected to posteriorization gradually during gastrulation. Transplantation of mesoderm cells from the lateral marginal to animal pole can transform the forebrain into hindbrain, and transplantation of yolk to animal pole of the blastoderm reverses AP polarity in the neuroectoderm [3], which suggests that the posterior signal including Nodal, Fgf, Wnt, and RA may reside in the non-axial mesoderm [4]. Fate map studies have revealed that hindbrain progenitors are located near the lateral marginal mesendoderm at early gastrulation and move toward the posterior dorsal midline through the convergent-extension movement. Thus, the geographically short distant to paraxial mesoderm facilitates hindbrain progenitor to receive the posterior transformation signal [5].

To maintain balance with neural posteriorization factors and retain the early-induced forebrain region, there must be some kinds of head organizers. In mouse, deletion of organizer gene *Lim1* or *otx2* results in ablation of head anterior to the otic vesicle [6]. Head organizer of zebrafish may lie in deep hypoblast cells of the shield and prechordal plate. The progenitor cells of prechordal plate migrate underneath the developing forebrain from its original embryonic shield region during the involution movement. It secrets not only

BMP antagonists, but also Wnt inhibitor dkk1, Cerberus, and Frzb. Dual inhibition of BMP and Wnt signal is important for proper head induction and central nervous system (CNS) AP patterning [7].

*Sox* genes encode a group of proteins that carry DNA-binding high-mobility group (HMG) domain and additional domains implicated in transcriptional regulation. They involve in various developmental processes in a manner influencing cell specification. *Sox31* is firstly isolated from *Danio rerio* cDNA library using *Drosophila* SoxNeuro as a probe [8]. It is considered as a maternal factor in oocyte and ubiquitously express in zebrafish neural system. Overexpression of *Sox31* leads to severe head patterning defects that are characterized by loss of intact eye structure. In case of *Sox31* knockdown, the posterior structure is truncated. Based on the phenotype, several molecular markers were applied to detect the AP patterning. Finally, we propose a model of Sox31 involving the dorsal-anterior synergistic development process.

## Materials and Methods

### Fish maintenance

The wild-type zebrafish embryos were obtained from natural crosses of fish with the AB genetic background, and raised under standard conditions. The development stage was determined by interval from the fertilization time point at 28.5°C and the morphological criteria [9].

### Plasmid construct

Total RNA was extracted from fish embryo collected at various specific development stages using Trizol (Invitrogen, Carlsbad, USA), and then reverse transcription reaction was performed using the Superscript III reverse transcription kit according to the manufacturer's instruction (Invitrogen). *Sox31* (GenBank accession No. NM_131702.1) cDNA fragment with complete coding region was amplified by RT-PCR using the cDNA product from 48-h embryos as a template. The primer pairs used for *Sox31* were: 5′-CGCGAATTCCATGATGTACAGCAT GATGGAGCACG-3′ (sense) and 5′-CGCCTCGAGTCA GATGTGAGTGAGGGGAACAGTT-3′ (reverse). *Eco*RI cleavage site (underlined) and *Xho*I (underlined) cleavage site were introduced to ligate the fragment into myc-PCS2+ vector. The C-terminal deletion form of *Sox31* encoding amino acid 1−149 was amplified from the PCS2+ to *Sox31* plasmid and subcloned into PCS2+. The primer pairs used were 5′-CGCGAATTCGATGATGTAC AGCATGATGGAGCACG-3′ (sense) and 5′-CGCCTCGAG CAACAAGTTGCCCGCTGCAAGAGGA-3′ (reverse). The EnR chimera construct was generated by inserting the C-terminal deletion fragment into *Cla*I/*Nco*I sites of PCS2+ to EnR (containing the amino acid 1−226 of

*Drosophila* engrailed protein) plasmid, and VP16 chimera construct was generated by inserting the C-terminal deletion fragment into *Nco*I/*Cla*I sites of PCS2+ to VP16 (containing the amino acid 412−490 of Herpes simplex virus protein I VP16) plasmid [10]. To construct the morpholino efficiency testing plasmid, a fragment of *Sox31* (−178 to +92 nucleotides covering the ATG starting codon) was amplified from the 12 h post-fertilization (hpf) cDNA, and inserted into the *Bam*HI and *Eco*RI sites (underlined) of PCS2+ to GFP (green fluorescent protein) plasmid. Primer pairs used were 5′-CGCGGATCCCTT TGAGATTTCCCTCATTGCTGCT-3′ (sense) and 5′-C GCGAATTCCCGCTGCCCGGAGGAGACATGCCCG-3′ (reverse). The whole GFP coding sequence was inserted into *Nco*I and *Xba*I sites of PCS2+ plasmid.

### Morpholino and mRNA microinjection

Antisense morpholinos were obtained from the Gene Tools. LLC (Philomath, USA). Sequence of translational blocking morpholino for *Sox31* was designed against the ATG translation starting region including partial 5′-UTR (untranslated region) sequence (5′-TACATCATGCCAC TTCTCGCTTTGA-3′). As a specificity control, 4mut-*Sox31* morpholino carrying four bases substitution with respect to wild-type sequence was used. Morpholinos were diluted to desired concentration in nuclease free water, and were injected at the 1−2-cell stage. For sense mRNA injection, capped mRNA was synthesized using the mMessage mMachine kit (Ambion, Austin, USA) following the manufacturer's instruction. A volume of 4−5 nl was injected into the yolk of the 1−2-cell stage embryo. *GFP* mRNA was usually used as a control.

### *In situ* hybridization

The localization of mRNA transcripts was determined by whole mount *in situ* hybridization with digoxigenin-labeled riboprobes. Templates were transcribed *in vitro* using SP6 or T7 polymerases (Roche, Basel, Switzerland) and resuspended in diethylpyrocarbonate water. Embryos injected with capped mRNA or morpholino were maintained at 28.5°C until reaching the desired developmental stages, and then were fixed by 4% paraformaldehyde in phosphate-buffered saline buffer overnight at 4°C. Other procedures were performed according to the standard protocol described previously [11]. All images were captured at room temperature using a camera (DP71; Olympus, Tokyo, Japan) on a microscope (SZX16, 1×; Olympus). The acquiring software was DP Controller and DP Manager (DP71; Olympus).

### Reporter gene assay

To examine the influence of *Sox31* on Wnt signal, HEK293T cells placed in 24-well plates were transfected with 250 ng plasmid in total, including 180 ng
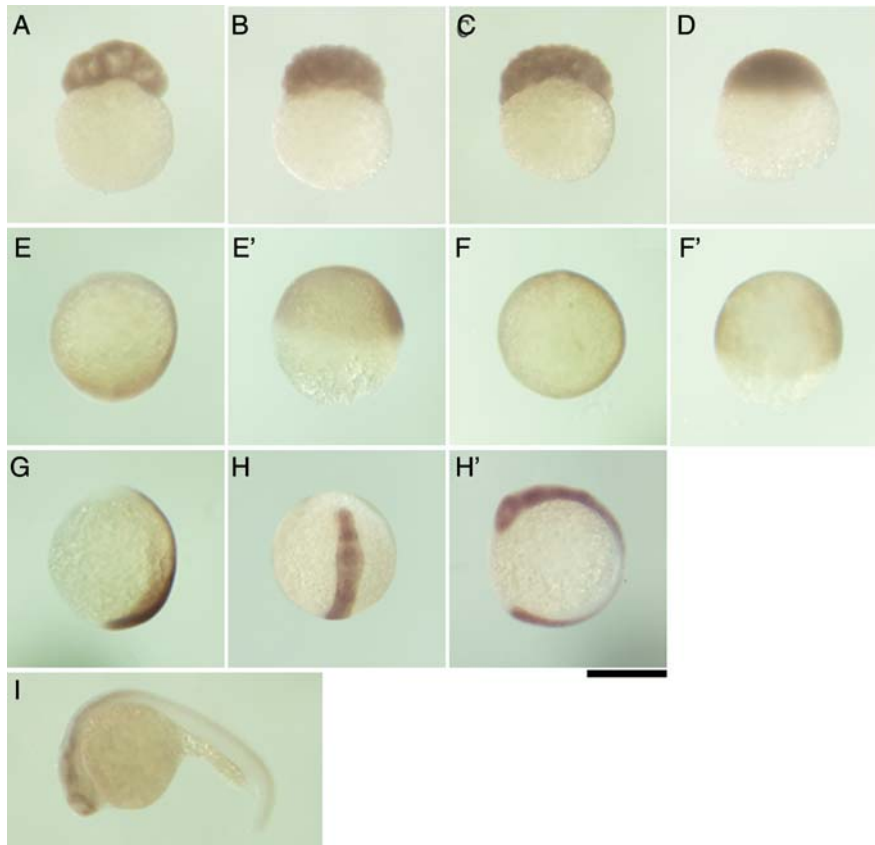
**Figure 1 *In situ* hybridization of *Sox31* during zebrafish early development**   Detection of *Sox31* signal respectively at 32-cell stage (A), 128-cell stage (B), 256-cell stage (C), and sphere stage (D). *Sox31* transcripts are uniformly distributed in the blastomere. (E, E′) At shield stage, *Sox31* is expressed in the future ectoderm. (F, F′) At 80% epiboly, *Sox31* is expressed at the dorsal side corresponding to the future neuroectoderm. (G) At tail bud stage, *Sox31* transcripts scatter in the neuroectoderm. (H, H′) At 10-somites stage, *Sox31* distributes along the neural tube, but none transcripts is detected in the optic vesicle. (I) At 24 hpf, *Sox31* is ubiquitously expressed in the midbrain and hindbrain, but only restricts in dorsal telencephalon and ventral diencephalon of forebrain. A–D, E′, F′, G, and H′ are shown by lateral view with dorsal toward the right. E and F are observed from the animal pole. I is photographed from lateral with anterior towards left. Scale bar=500 μm.

*Sox31*-PCS2+ plasmid or *lacZ* control plasmid, 20 ng TopFlash or FopFlash plasmid, and 50 ng GFP plasmid. In further dose-dependent assay, 200 ng plasmids were transfected in total: 15 ng TopFlash, 30 ng GFP, a serial gradient dose of *Sox31*-PCS2+, and *lacZ* plasmids. After 18 h of transfection, cells were treated with Wnt-3a conditioned medium or control medium for additional 6 h [12]. Cells were then lysed and luciferase assays were performed. In ΔN-β-catenin co-transfection assay, 5 ng of ΔN-β-catenin was co-transfected with 30 ng GFP, 15 ng TopFlash, and a gradient dose of *Sox31*-PCS2+. The luciferase activities presented were normalized against the levels of GFP expression as described previously.

## Results

### Spatial and temporal expression pattern of *Sox31* during zebrafish early embryogenesis

To explore the functional role of *Sox31* during zebrafish embryogenesis, we examined the localization of its transcripts corresponding to key development time points.

Expression of *Sox31* was firstly detected as early as the 32-cell stage. The results showed that *Sox31* was distributed globally during early blastula stage [**Fig. 1(A–C)**]. Following mid-blastula transition (MBT), zygotic *Sox31* ubiquitously expressed throughout the blastomere, but slightly weaker in the dorsal blastoderm margin at sphere stage [**Fig. 1(D)**]. At shield stage, *Sox31* transcripts scattered around the future ectoderm in a circular manner [**Fig. 1(E,E′)**]. As convergence movement during gastrulation, *Sox31* gradually condensed toward the dorsal side, which would develop into neuroectoderm [**Fig. 1(F,F′)**]. Till the completion of gastrulation, *Sox31* was universally distributed in the neural plate [**Fig. 1(G)**]. During somitogenesis, the expression of *Sox31* along the developing neural tube was more regionalized. It was robustly detected in the CNS, including forebrain, midbrain, and hindbrain region at 10-somites stage, but an absence of its expression in the optic vesicle was also observed [**Fig. 1(H,H′)**]. At 24 hpf, the subdivision of the CNS was more explicit. In the forebrain region, *Sox31* was only expressed in dorsal telencephalon and ventral diencephalon, but excluded from
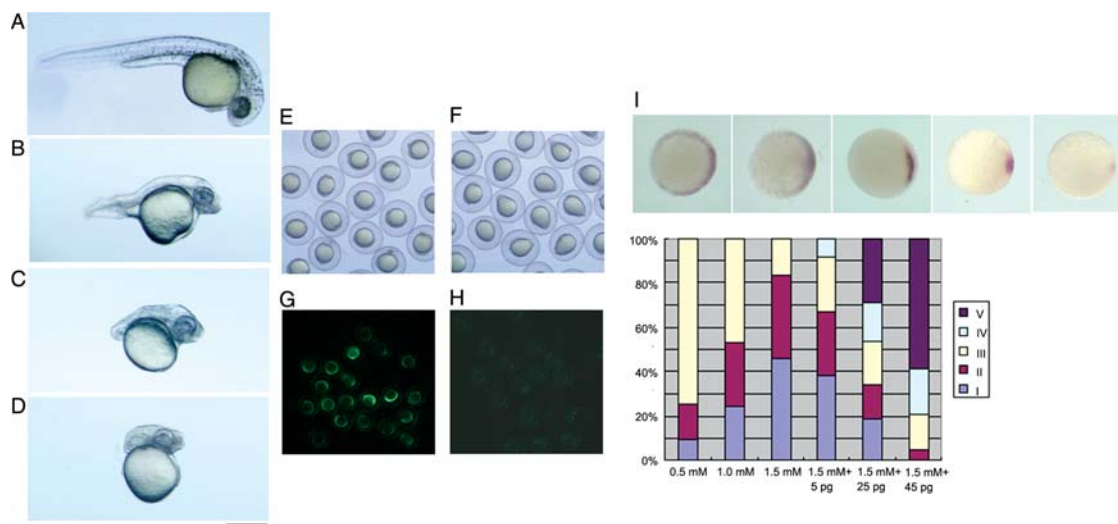
**Figure 2 Abnormality caused by *Sox31* translational blocking morpholino (MO) can be rescued by MO insensitive mRNA** (A) Normal control at 30 hpf. (B–D) Classification of abnormal phenotype caused by *Sox31* MO. Ranging from C1 to C3, embryos gradually lost its posterior structure with enlarged head (n = 28/80, 22/80, 19/80). (E–H) *Sox31* MO was co-injected with MO efficiency testing construct. Under the light microscope, *Sox31* morphants is oval shaped (F) relative to the normal embryos (E). *Sox31* MO can successfully block the GFP fluorescence (H), while the mismatched MO does not (G). (I) Number of embryos with altered *gsc* expression pattern in *Sox31* MO injection and mRNA rescue group are shown by percentage value. Scale bar=500 μm.

the ventral telencephalon and eye region. Posteriorly in the midbrain and hindbrain, it was uniformly distributed [**Fig. 1(I)**]. The expression pattern indicates that *Sox31* is a maternal supplied factor and may be involved in the neural development process.

## Knockdown of *Sox31* leads to dorsalized phenotype with expansion of anterior neural fates

To investigate the function of *Sox31 in vivo*, we designed an antisense morpholino oligonucleotide, which could specifically bind to the translational start region of *Sox31* and sequentially block the translation process. A dose-dependent assay was performed, using a four-base mismatched morpholino as a negative control. As little as 0.5 mM morpholino led to apparent dorsalized phenotype, which could be classified into classes ranging from C1 to C3 according to the abnormal phenotypic severity: the weak and intermediate phenotype consisted of shortened and twisted tail, and lost its trunk region gradually with a slightly enlarged head region; the severe phenotype displayed total loss of tail and trunk region, and enlargement of telencephalon and eyes [**Fig. 2(A–D)**]. Additionally, to test the efficiency of *Sox31* morpholino, its translation initiation region including morpholino binding site was cloned and inserted upstream of GFP coding region. mRNA product (50 pg) transcribed from the corresponding region of the construct was co-injected into one-cell stage embryo together with 0.8 mM morpholino. At the end of gastrulation, *Sox31* morpholino-injected individuals exhibited ovary shape, and GFP fluorescence was completely blocked compared with controls, which supported the

validity of the morpholino we applied [**Fig. 2(E–H)**]. The *gsc* gene normally expressed at dorsal shield region was selected as a marker, which indicated the extent of dorsalization in rescue experiment [**Fig. 2(I)**]. *Sox31* morpholino led to *gsc* expansion in a dose-dependent manner, whereas co-injection with morpholino-insensitive *Sox31* mRNA could gradually reverse *gsc* expansion phenotype. Excessive mRNA will elicit dorsal repression.

Based on the phenotypic evidence, several molecular markers that were critical for normal neuroectoderm patterning and mesoderm development were examined. *Otx2* [13] is important for early neural specification, and its expression has been detected in the presumptive brain-midbrain [12]. In morphants, expression domain of *otx2* was posteriorly shifted, with ventral laterally expansion from the dorsal side [**Fig. 3(A,B)**]. To get a more comprehensive view of the altered neuroectoderm AP patterning caused by *Sox31* morpholino, we carried out double and triple staining. *Rx3* [14] is normally expressed in the anterior most neural plate that gives rise to the forebrain and retinal tissues; *Pax2a* [15] represents the mid-hindbrain boundary, and *hoxb1b* is a hindbrain marker gene. In double staining of *rx3* and *pax2a*, both of them ventral-posteriorly expanded [**Fig. 3(E,F)**]. We further adopted *hoxb1b* [16] in triple staining together with *rx3* and *pax2a*. The expression of forebrain marker was enhanced, and caudally shifted at the expense of sharp reduction of hindbrain marker [**Fig. 3(C–D′)**]. An organizer gene, *gsc* expressed at dorsal shield region and future prechordal plate [17] was examined at 6 hpf. In *Sox31* loss-of-function individuals,
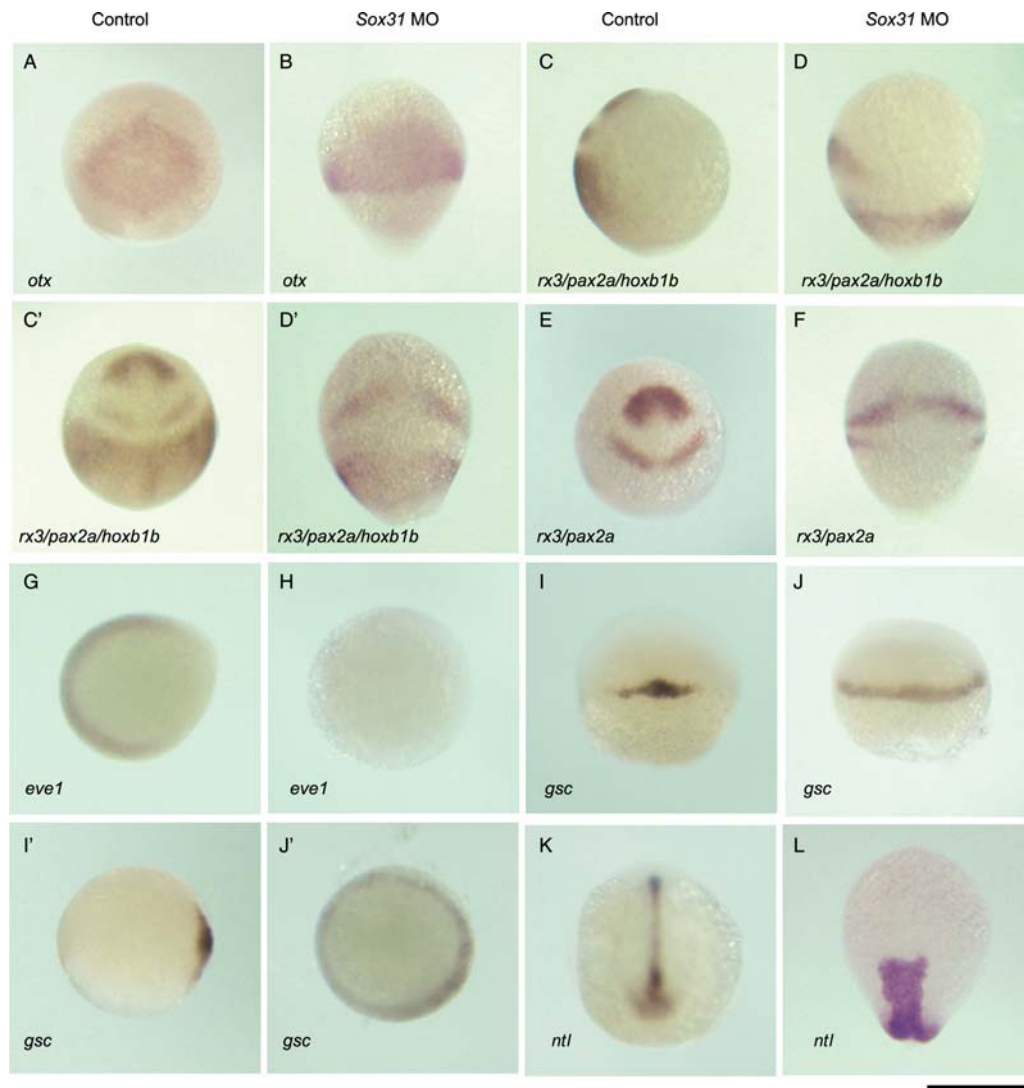
**Figure 3 Altered expression of marker genes correlates with neuroectoderm AP patterning and organizer formation due to *Sox31* loss-of-function** (A) Control embryo showing normal *otx2* expression at 10 hpf. (B) Ventral lateral expansion of *otx2* in *Sox31* morphants ($n = 50/58$). (C–D′) Triple staining of *rx3/pax2a/hoxb1b*. Compared with control embryos (C, C′), *rx3* expression in morphants posteriorly expands, with sharp reduction of *pax2a* and *hoxb1b* ($n = 59/70$) (D, D′). Double staining of *rx3/pax2a* in control embryos (E) and *Sox31* morphants ($n = 35/48$) (F). Embryos were fixed at 12 hpf in double and triple staining. Detection of ventral marker *eve1* at 50% epiboly in control embryos (G) and *Sox31* morphants ($n = 48/55$) (H). (I, I′) Control embryos show normal expression of *gsc* at germ ring stage. (J, J′) Expression of *gsc* is significantly expanded towards ventral lateral side in *Sox31* morphants ($n = 25/53$). (K) Normal expression of *ntl* at 10 hpf. (L) Expression of *ntl* shows a shortened and laterally expanding manner in *Sox31* MO injected embryos ($n = 38/50$). A, B, C′–F, I, J, K, L are shown by dorsal view, C–D are shown by lateral view, and G–H, I′–J′ are shown by animal pole view. Scale bar＝500 μm.

its expression domain expanded toward the ventral side in a circular manner, a typical phenomenon of dorsalization [**Fig. 3(I–J′)**], while ventral marker *eve1* [18] completely lost its expression in morphants [**Fig. 3(G,H)**]. At 10 hpf, *ntl* [19] mainly expressed in notochord structure. In morphants, it exhibited shortened and laterally expansion pattern [**Fig. 3(K,L)**]. Taken together, loss of function of *Sox31* greatly promotes dorsal-anterior fates.

To further confirm the phenotypic defects caused by *Sox31* loss of function, we adopted another knockdown strategy using a dominant negative (DN) construct of *Sox31*. The N-terminal region of *Sox31* including its HMG box was cloned from whole sequence, deleting the C-terminal region [20,21] [**Fig. 4(A)**]. Such a DN construct could antagonize the function of endogenous *Sox31* by competing for the same binding sites but incapable of affecting transcription. Overexpression of its mRNA also led to dorsalization phenotype. The loss-of-function individuals displayed shortened body axis, twisted tail, and protruding telencephalon marked by black triangle [**Fig. 4(B,C)**]. The brain-midbrain boundary (MHB)
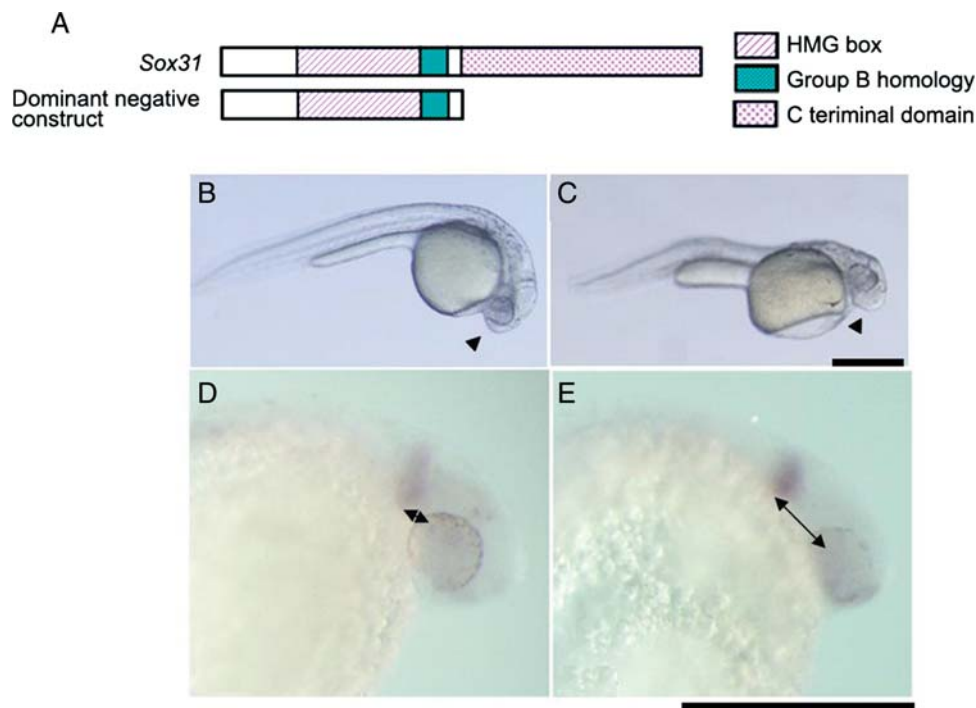
**Figure 4 Ectopically expression of a DN form of *Sox31* phenocopies morpholino effects** (A) Schematic representation of DN construct for *Sox31*, in which the C-terminal domain was deleted. (B, C) Phenotypic characteristic caused by DN construct ($n = 39/46$), with respect to control embryo at 24 hpf. Black triangle indicates telencephalon. (D–E) Of DN construct overexpression embryos, MHB boundary marked by *pax2a* is significantly posterior shifted ($n = 50/55$) (E), which indicates the enlarged forebrain by contrast to control embryos (D). Arrowhead marks the distance from MHB to eye. Embryos were fixed at 24 hpf. Scale bar=500 μm.

posteriorly shifted from its original position proximal to eye, which was demonstrated by *pax2a* staining [**Fig. 4(D,E)**]. Thus, *Sox31* may play an essential role in limiting the anterior neural territory.

## Overexpression of *Sox31* results in loss of anterior structure and impaired organizer formation

To verify the results from the loss-of-function study, we further performed gain-of-function experiments by injecting *Sox31* mRNA into zebrafish embryo. About 50 pg/embryo led to 40%–50% lethality, and the rest of the alive embryos were categorized into three classes according to the severity of abnormality: the elementary phenotypic defects was described as cyclopia, twisted notochord and shortened body axis; the intermediate phenotype was characterized by a complete loss of anterior head and eye structure together with an absence of notochord; at its most extreme, the severely affected embryos lacked any distinguishable dorsoventral axis or AP characteristic, thus a totally disrupted body pattern [**Fig. 5(A–D)**]. At 10-somites stage, we observed embryos under the light microscope and focused on its eye development. *Sox31* overexpression led to significant reduction or absence of the eye, which represented the most anterior neural tissue [**Fig. 5(E,G)**].

The phenotype from *Sox31* gain-of-function study was further examined by molecular marker, which indicates the

regionalization of neuroectoderm and dorsal mesoderm development. *Six3* is a homeobox gene expressed in the anterior neural plate where the optic vesicle and forebrain develop at 10 hpf [22]. In *Sox31* ectopically expressed individuals, *six3* expression domain sharply reduced or completely lost, consistent with *rx3* staining result [**Fig. 6(A–D')**]. *Otx2*, representing the forebrain and midbrain development, also exhibited a more restrained expression territory [**Fig. 6(E,F)**]. Midbrain marker *eng2* [23] and MHB marker *pax2a* were analyzed in 12 hpf embryos. *Sox31* misexpression led to anterior-toward migration of these two genes [**Fig. 6(G–J)**]. *Hoxb1b*, a hindbrain tissue indicator, also displayed a similar anterior expansion characteristic compared with controls [**Fig. 6(M,N)**]. In *rx3/pax2a* double staining assay, the relative occupying domain of forebrain versus posterior brain was reversed opposite to *Sox31* morphants [**Fig. 6(K,L)**], which enhanced the role for *Sox31* as a forebrain fates repressor.

Additionally, ectopic *Sox31* led to twisted or shortened notochord with *ntl* expression only detected in the tail bud [**Fig. 6(Q–R')**]. Somites generation marked by *myoD* [24] was also severely disrupted [**Fig. 6(O,P)**]. Organizer gene *gsc* was repressed or abrogated from the embryonic shield [**Fig. 6(S–T')**]. In summary, increase of *Sox31* level results in a dose-dependent loss of anterior head tissue along with
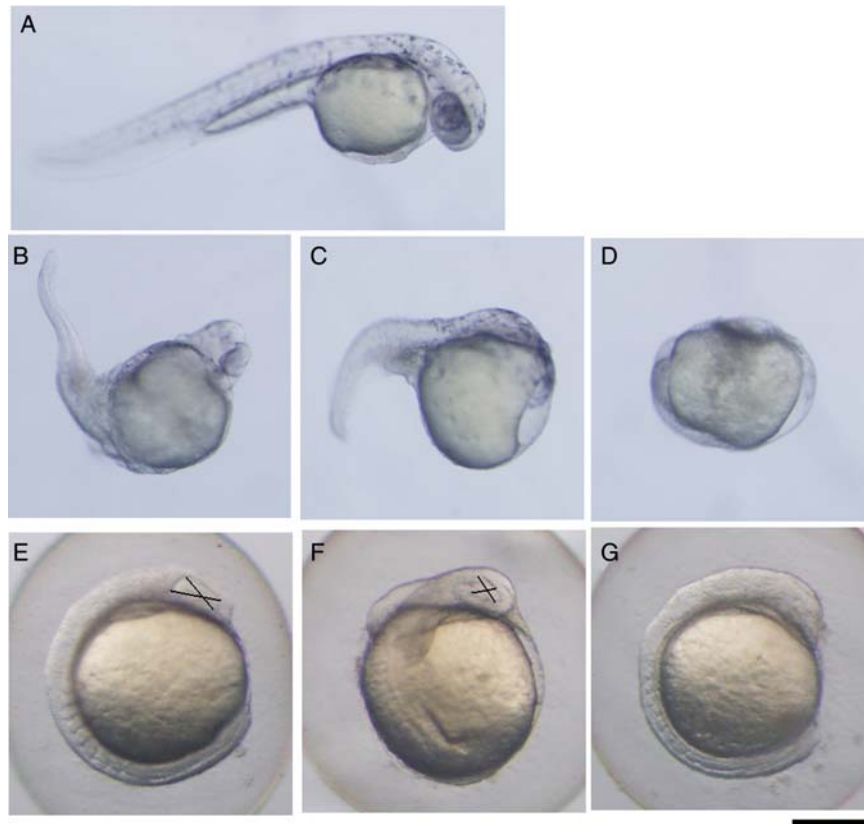
**Figure 5  Phenotype of *Sox31* mRNA injected embryos**   (A) Control embryos under the light microscope at 30 hpf. (B−D) Phenotypic classification. About 50 pg *Sox31* mRNA was injected per embryo. The affected embryos gradually lost its anterior structure and exhibiting severe axis defects ($n = 15/86$, $18/86$, $10/86$, respectively, for B, C, and D). (E) Normal control at 10-somites stage. (F−G) *Sox31* overexpression embryos display a significantly reduced eye region or completely lost of both eyes ($n = 28/74$, $20/74$). Scale bar=500 μm.

anteriorly expansion of posterior structure, whereas the disrupted dorsal mesoderm implies that the organizer activity may have been impaired.

### Sox31 functions as a transcriptional repressor *in vivo*

To clarify the molecular nature of Sox31 as a transcriptional factor, we generated chimeric constructs by fusing N-terminal region of Sox31 (aa 1−149, including its HMG box) to the repressor domain of *Drosophila* engrailed protein or transcriptional activation domain of Herpes simplex virus VP16 protein [10] [**Fig. 7(A)**]. Sox31-EnR not Sox31-VP16 construct strongly mimicked the activities of wild-type Sox31, which resulted in embryos with various degrees of dorsal-anterior defects ranging from class 1 to class 4 [**Fig. 7(B−F)**]. The weakly affected individuals displayed cyclopia and shortened body axis. With the phenotype went more severe, the abnormal embryos gradually lost both eyes or entire anterior head, exhibited abnormal chordamesoderm or completely disrupt body pattern. Phenotypes caused by EnR and VP16 chimera were then elucidated by molecular markers. The forebrain tissue stained by *rx3* and *otx2* was significantly repressed by Sox31-EnR chimera overexpression

[**Fig. 7(H−M)**]. Consistent with the alteration of forebrain patterning, *gsc* was also inhibited by Sox31-EnR chimera [**Fig. 7(N−P)**]. Notochord development indicated by *ntl* was severely disrupted by EnR fusion resembling the effect of wild-type Sox31 [**Fig. 7(Q,S)**]. These results imply that Sox31 may act as a transcriptional repressor rather than an activator during dorsal-anterior specification process.

### Sox31 negatively regulates β-catenin-mediated transcriptional activity

The gene *boz* was identified to have the ability to induce dorsalization of the embryo. Evidence from gain-of-function and loss-of-function experiments implies that *Sox31* acts as a repressor of dorsal-anterior determination, which raises our curiosity to inquiry into the relationship between *boz* and *Sox31*. Results from the *in situ* hybridization, *boz* transcripts in the dorsal blastoderm margin were sharply diminished or completely vanished from its usual expression territory at 4 hpf resulting from the ectopically expressed *Sox31* [**Fig. 8(B,B′,E)**]. By contrast, *boz* expression in *Sox31* morphants was enhanced, and tended to laterally expand from the most dorsal side [**Fig. 8(C,C′,F)**].
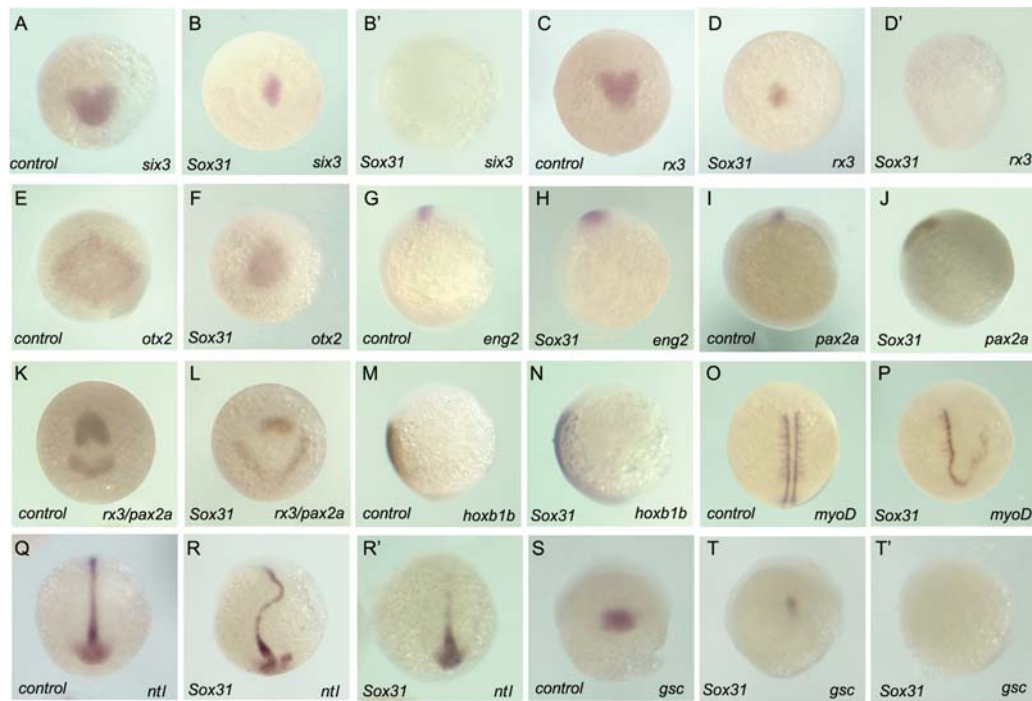
**Figure 6 Ectopically expression of *Sox31* results in dorsal-anterior inhibition**   Control embryos showing normal expression of *six3* (A) and *rx3* (C) at 10 hpf. Reduction or completely loss of *six3* expression in the forebrain region (*n* = 25/65, 30/65) (B, B′) and *rx3* in retinal tissue (*n* = 36/73, 28/73) due to *Sox31* overexpression (D–D′). (E) Normal expression pattern of *otx2* at 10 hpf. (F) Repressed *otx2* expression in forebrain/midbrain region caused by ectopically expressed *Sox31* (*n* = 42/53). Compared with reduced forebrain tissue, the posterior brain marker *eng2*, *pax2a* and *hoxb1b* anteriorly expand (*n* = 30/45, 26/33, 50/58) (H, J, N) relative to normal control (G, I, M). For *eng2*, *pax2a*, and *hoxb1b*, embryos were fixed at 12 hpf. (K) Normal expression of *rx3/pax2a* is shown by double staining at 12 hpf. (L) Expanded *pax2a* and reduced *rx3* is apparent in *Sox31* overexpressed embryos (*n* = 30/43). Mesodermal markers were examined at 10 hpf. Normal expression of *myoD* and *ntl* are shown in (O) and (Q), respectively. Abnormal somites are indicated by *myoD* due to *Sox31* mRNA (*n* = 50/65) (P). Notochord development exhibits a twisted or severely shortened pattern (*n* = 32/55, 12/55) (R–R′). Organizer marker gene *gsc* is reduced or lost due to ectopic *Sox31* (*n* = 46/76, 18/76) (T–T′) with respect to normal control (S), embryos were fixed at 6 hpf. A–F, K–L are shown by dorsal animal pole view, G–J, M–N are shown lateral view, and O–T′ are observed from the dorsal side. *Sox31* mRNA (50 pg) was injected per embryo. Scale bar=500 μm.

*Boz* is a direct target gene of pre-MBT Wnt/β-catenin pathway, which contains nine putative Tcf/Lef-binding sequences within its promoter region [25]. To investigate whether *Sox31* was involved in Wnt/β-catenin pathway, a pair of reporter constructs, TopFlash and FopFlash [26], were used to evaluate effects of *Sox31* on Tcf/Lef-dependent transcriptional activity. TopFlash contains three optimal copies of Tcf/Lef-binding sites upstream of a minimal thymidine kinase promoter directing transcription of a firefly luciferase gene. FopFlash with mutated copies of Tcf/Lef-binding sites is used for measuring the unspecific transactivation or repression. We co-transfected Top or Fop reporter with *Sox31* into HEK293T cells and found that Wnt3a stimulated TopFlash activity was significantly suppressed by *Sox31*, whereas FopFlash activity showed no difference from the *lacZ* control [**Fig. 8(G)**]. Additionally, *Sox31* inhibited Wnt3a-triggered TopFlash activity in a dose-dependent manner, as low as 5 ng would take effects [**Fig. 8(H)**]. To further elucidate whether *Sox31* exerted its influence on TopFlash activity at the

transcriptional level or by affecting the stabilization of β-catenin, we introduced a modified β-catenin construct that lacks its N-terminal region. It does not undergo the ubiquitination-dependent degradation and can accumulate in the nucleus even without Wnt signal triggering. TopFlash activity was suppressed in a dose-dependent manner by co-expression of ΔN-β-catenin and *Sox31*, which excluded the possibility that stabilization of β-catenin may be affected [**Fig. 8(I)**]. Taken together, *Sox31* can specifically repress the Tcf/Lef-dependent transcriptional activity, and it is a possible way through which *Sox31* regulates the expression level of *boz*.

### Sox31's influence on CNS AP patterning is related with post-MBT Wnt signal

In *Sox31* morphants, the posterior structure MHB stained by *pax2a* was greatly compromised, and the hindbrain development marked by *hoxb1b* was also suppressed [**Fig. 3(C–F)**]. To verify whether the reduced posterior neural structure was relevant to altered Wnt signal, we
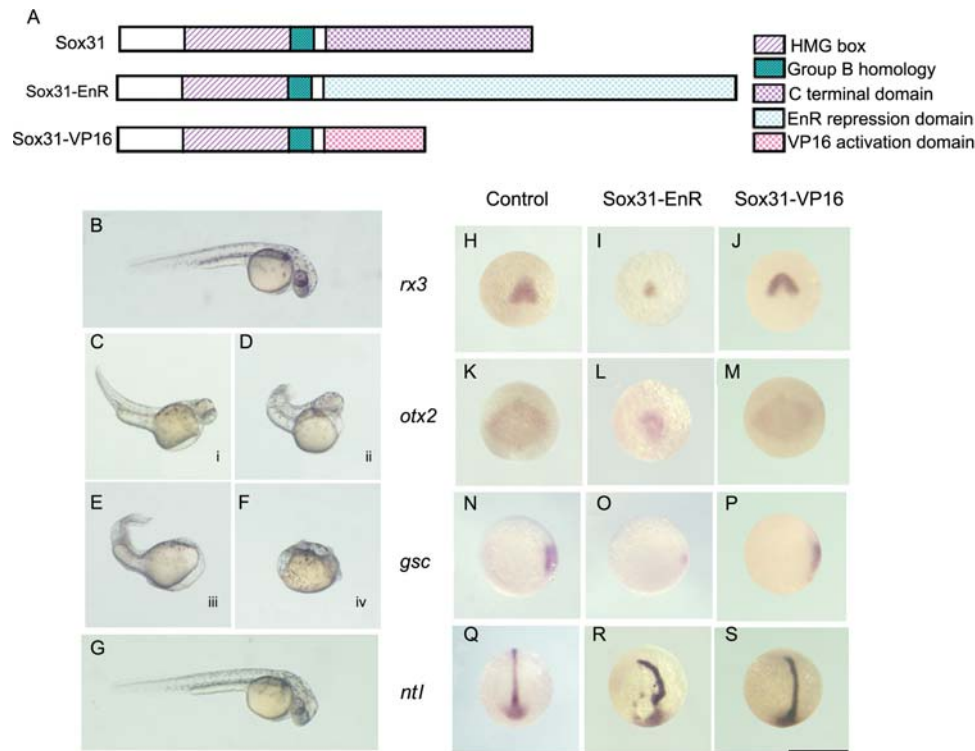
**Figure 7 Sox31-EnR fusion construct mimics *Sox31* effects when overexpressed** (A) Schematic drawing of Sox31-EnR and Sox31-VP16 chimera constructs, in which the C-terminal deletion fragment of *Sox31* was placed upstream of EnR domain and VP16 domain, respectively. (B) Control embryo at 30 hpf. (C–F) Categorization of different phenotype elicited by Sox31-EnR construct. From grade I–IV, the abnormality goes more severe. Embryos gradually lost dorsal-anterior structure (*n* = 15/75, 20/75, 16/75, 10/75). mRNA (200 pg) was injected into 1-cell embryo. (G) Sox31-VP16 mRNA injected embryo (*n* = 45/50). Forebrain patterning was analyzed by *rx3* and *otx2*. Normal expression is shown in (H) and (K), respectively. Expression of *rx3* and *otx2* is repressed by Sox31-EnR construct (*n* = 42/53, 34/50) (I, L), but tend to be normal in Sox31-VP16 overexpression group (*n* = 30/35, 42/50). Dorsal mesoderm development was detected by *gsc* and *ntl*. Normal expression of *gsc* is shown in (N) and *ntl* in (Q). Due to effect of Sox31-EnR, the organizer tissue is significantly repressed (*n* = 38/45) (O), and the notochord displays a shortened and twisted pattern (*n* = 40/58) (R). Their expression pattern in Sox31-VP16 expression embryo is similar to wild type (*n* = 42/42, 50/53) (P, S). Scale bar=500 μm.

checked the expression of *Wnt8* and its target T-box gene *tbx6* at 6 hpf [27]. As our speculation, *Wnt8* disappeared from its usual dorsal-lateral expression territory and was restrained to the more ventral side [**Fig. 9(A,B)**]. Similar to *Wnt8*, ventral gene *tbx6* was also reduced, completely cleared from the blastoderm margin [**Fig. 9(C,D)**], which suggests the reduction of the posterior neural structure in *Sox31* morphants is bona fide a Wnt signal-related phenomenon. On the contrary, overexpression of *Sox31* led to severe anterior head defects. As is hinted by previous study, the axial mesoderm derivative structure prechordal plate acts as an important anterior head organizer, and thus we fixed *Sox31* overexpressed embryos at 90% epiboly stage to detect prechordal plate development marked by *gsc*. It was found that *gsc* expression region was greatly diminished and posteriorly placed compared with normal control, which indicated the severely impaired prechordal plate [**Fig. 9(E–F′)**]. Moreover, *ntl* is a pan-mesoderm marker expressed around the germ ring at 80% epiboly. Due to *Sox31* overexpression, *ntl* only retained in the ventral lateral mesoderm region and its expression in this region was enhanced to some extent, while was lost its dorsal appearance [**Fig. 9(G,H)**]. In conclusion, loss of dorsal most mesoderm and defects in prechordal plate probably result in insufficient repression of paraxial mesoderm signal that leads to reduction of the developing forebrain tissue.

*Dkk1* encoding a Wnt inhibitor is secreted by the prechordal plate [28,29]. Microinjection of 100 pg of *Sox31*/ embryo led to high lethality, whereas co-injection of 12.5, 25 and 40 pg *dkk1*, respectively, with 100 pg of *Sox31* could gradually suppress the high death frequency and significantly restored normal embryo ratio [**Fig. 9(I)**]. More than 40 pg *dkk1* co-injection led to apparent dorsalization phenotype. *Dkk1* is effective to reverse the lethality and defects caused by *Sox31* overexpression.

## Discussion

Fate mapping in zebrafish reveals cells contributing to major subdivision of nervous system occupy distinct regions at the onset of gastrulation. Explants from lateral
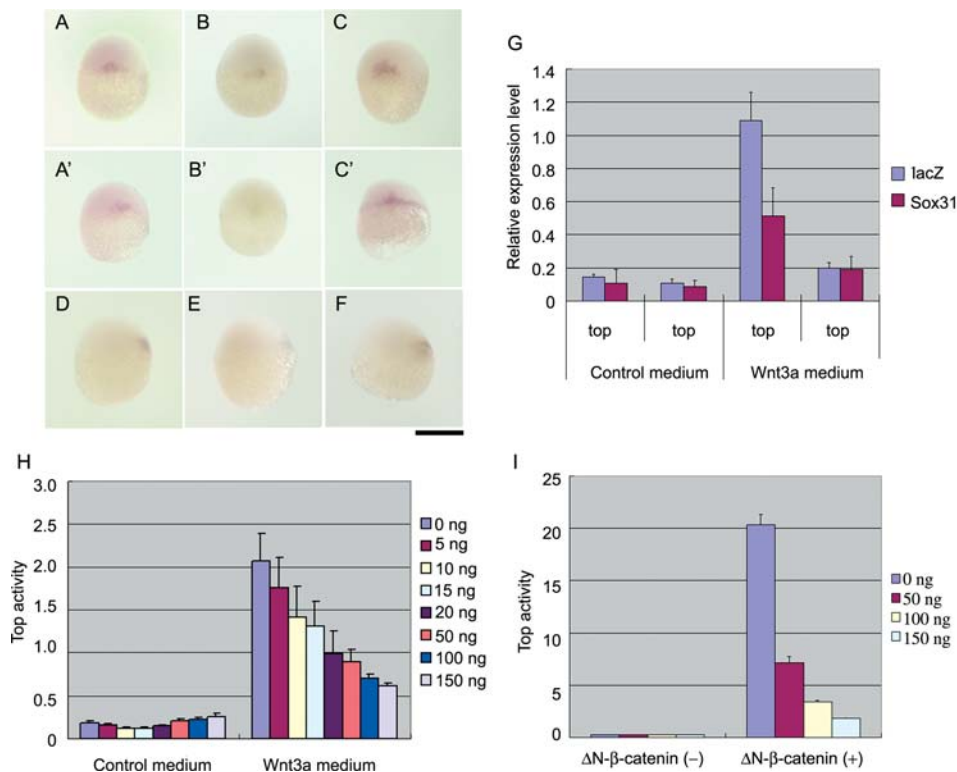
**Figure 8 Sox31 negatively regulates *boz* expression and activity of TopFlash/FopFlash luciferase reporter** (A, A′) Normal expression of *boz* at sphere stage. (B, B′) *Boz* is repressed or lost in *Sox31* mRNA overexpression embryos (*n* = 26/65, 19/65). (C, C′) *Boz* expression is enhanced to various extent due to *Sox31* MO (*n* = 23/50, 15/50). Lateral view of normal and altered *boz* expression due to *Sox31* mRNA or MO are shown in (D–F) corresponding to the above figure. Embryos were fixed at 4 hpf. (G) *Sox31* was overexpressed in HEK293T cells with TopFlash or FopFlash reporter plasmids. Eighteen hours after transfection cells were treated with Wnt3a medium or control medium for additional 6 h, and then luciferase activity was determined. *Sox31* can significantly repress the Top activity stimulated by wnt3a medium, whereas the Fop activity is not affected. (H) Sox31 suppresses Top activity in a dose-dependent manner. (I) *Sox31* can also repress Top activity in a dose-dependent manner when co-overexpressed with ΔN-β-catenin. Scale bar=500 μm.

regions of 6-h embryos, corresponding to the hindbrain region of the fate map, express *gata3* (*gta3)*, a marker of non-neural ectoderm, *opl* and *fkd5*, instead of *krx20* [30]. This is consistent with the view that induced neural tissue develops with anterior identity, which then is transformed to more posterior identity. Despite the prevailing idea that anterior neuroectodermal fate is determined prior to posterior neuroectodermal, it is not known which genes help to subdivide distinct parts of CNS. In our study, we observed that regions normally belonging to forebrain and mid-hindbrain territory were rearranged due to the altered level of *Sox31*. In embryos expressing excessive *Sox31*, the forebrain fates were significantly repressed, typically represented by reduced or loss of eye structure, whereas the hindbrain structure exhibited anteriorly expansion. Oppositely, in *Sox31* morpholino-injected embryos, region occupied by forebrain structure was enlarged and ventral posteriorly shifted, compared with the remarkably suppressed hindbrain [**Fig. 10(A)**]. To exclude the possibility that the enhanced forebrain fates in *Sox31* morphants were secondary effects of dorsalization, we further adopt a

dominant-negative method. The extent of phenotypic defect caused by such a DN construct was slightly differed from that elicited by morpholino, but the effects on CNS AP patterning were also apparent. *Pax2a* indicated the MHB was normally positioned proximal to eye, and the territory in the front of it could be defined as forebrain's. Due to loss-of-function resulting from the DN construct, MHB was displaced posteriorly and further away from its original region, corresponding to the enlarged forebrain. In conclusion, our results support the two-step transformation model that the final territory distributed to forebrain and hindbrain along the CNS AP axis is determined by the mutual repression activity of the patterning signal. If the forebrain patterning signal is compromised, there will be insufficient repression upon the posterior transformation signal, which finally leads to excessive hindbrain structure encroaching upon the forebrain territory, and vice versa. *Sox31* acts as an important determinant during such regionalization process in zebrafish.

MBT is a vital development period in many animals, during which the early maternal mRNAs undergo
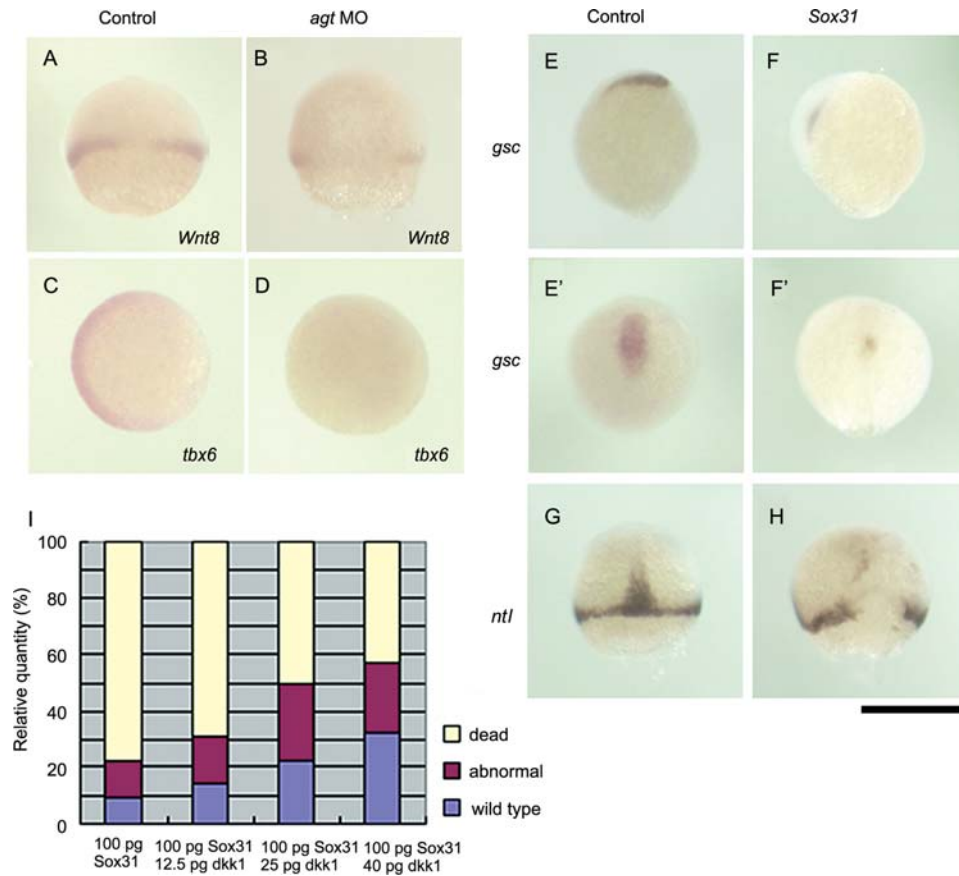
**Figure 9 Relationship between *Sox31* and post-MBT Wnt signal** (A) Normal expression of *wnt8* in control embryos. It is only excluded from the dorsal-most side. (B) In *sox31* MO injected embryos, *Wnt8* is cleared from the dorsal lateral side and restrained more ventrally (*n* = 52/61). *Wnt8* target gene *tbx6* completely lost its expression due to decreased *Sox31* level (*n* = 49/56) (D) compared with normal control (C). Embryos were fixed at 6 hpf. (E, E′) Lateral and dorsal view of normal prechordal plate patterning indicated by *gsc* at 90% epiboly stage. (F, F′) Prechordal plate patterning in *Sox31* overexpression embryos, shown by lateral and dorsal view, respectively (*n* = 50/55). (G) Normal *ntl* expression at 80% epiboly stage. (H) *Ntl* lost its dorsal most existence due to *Sox31* overexpression (*n* = 60/72). (I) *Dkk1* could rescue the abnormality and lethality caused by *Sox31* in a dose-dependent manner. *Sox31* mRNA (100 pg) was co-injected in rescue experiments. Scale bar=500 μm.

degradation while the zygotic genome activates [31]. It also acts as a special time point for body axis patterning. Before MBT, β-catenin accumulates on the future dorsal side and activates a series of zygotic organizer genes including *boz*, *dkk*, and *sqt*. Among them, *boz* functions as an important upstream organizer gene to initiate dorsal specification. We found that the expression level of *boz* was suppressed by ectopic *Sox31*, and strengthened by knockdown of it. In Top/Fop luciferase assay, *Sox31* could specifically repress β-catenin/Tcf/Lef-mediated transcriptional activity, which was probably the way that *Sox31* affected *boz* expression and dorsal mesoderm development. The existence of *Sox31* in pre-MBT embryo as a maternal factor enables it to take part in early dorsal specification events.

Later after MBT, Wnt signaling appears to be involved in AP patterning of the neural axis. Functionally knockdown of *Wnt8* leads to severe ventral posterior defects together with expanded dorsal and forebrain tissue, indicating the role of post-MBT Wnt signal in promoting the

posterior neural fates [32]. In our study, knockdown of *Sox31* led to reduced Wnt signal corresponding to suppressed hindbrain structure, whereas the lethality and prechordal plate patterning defect caused by ectopic *Sox31* could be rescued by Wnt antagonist *dkk1*. All these results suggest that the CNS AP patterning defect elicited by *Sox31* is related with post-MBT Wnt signal.

Boz acts as a central molecular that links both the pre- and post-MBT Wnt signal. It is induced by pre-MBT β-catenin, and it can reciprocally inhibit post-MBT Wnt signal. In *boz* mutant, embryos lack organizer derivatives and exhibit reduced neural tissue, which is characterized by lack of forebrain structures and expansion of midbrains and hindbrains [33]. As shown above, it is reasonable that *Sox31* negatively regulated *boz* expression, through which it indirectly activated the post-MBT Wnt signal. The excessive Wnt signal in turn transformed forebrain and midbrain toward hindbrain fates, which led to the forebrain patterning defects observed in *Sox31* overexpression embryos [**Fig. 10(B)**]. For a long time, whether the AP patterning of

**Figure 10 Schematic representation of *Sox31*'s influence on neuroectoderm AP patterning (A) and model for *Sox31* coordinating the organizer specification and neural regionalization (B)** A, anterior; P, posterior; t, telencephalon; r, retinal; d, diencephalon; m, midbrain; h, hindbrain; D, dorsal; V: ventral.

neuroectoderm is dependent on organizer activity is a controversial issue. *Sox31* functions as an essential factor that coordinates dorsal-anterior development in early zebrafish embryo, and enhances the relationship between them.

## References

1 Schier AF and Talbot WS. Molecular genetics of axis formation in zebrafish. Annu Rev Genet 2005, 39: 561–613.

2 Gamse J and Sive H. Vertebrate anteroposterior patterning: the Xenopus neurectoderm as a paradigm. Bioessays 2000, 22: 976–986.

3 Woo K and Fraser SE. Specification of the zebrafish nervous system by nonaxial signals. Science 1997, 277: 254–257.

4 Kudoh T, Wilson SW and Dawid IB. Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 2002, 129: 4335–4346.

5 Woo K and Fraser SE. Specification of the hindbrain fate in the zebrafish. Developmental Biology 1998, 197: 283–296.

6 Matsuo I, Kuratani S, Kimura C, Takeda N and Aizawa S. Mouse Otx2 functions in the formation and patterning of rostral head. Genes Dev 1995, 9: 2646–2658.

7 Niehrs C. The Spemann organizer and embryonic head induction. EMBO J 2001, 20: 631–637.

8 Girard F, Cremazy F, Berta P and Renucci A. Expression pattern of the Sox31 gene during zebrafish embryonic development. Mech Dev 2001, 100: 71–73.

9 Lv WJ, Zhang YB, Wu ZL, Chu L, Koide SS and Chen YG. Identification of *WSB1* gene as an important regulator in the development of zebrafish embryo during midblastula transition. Acta Biochim Biophys Sin 2008, 40: 478–488.

10  Bae YK, Shimizu T, Yabe T, Kim CH, Hirata T, Nojima H and Muraoka O, *et al*. A homeobox gene, pnx, is involved in the formation of posterior neurons in zebrafish. Development 2003, 130: 1853–1865.

11  Thisse C, Thisse B, Schilling TF and Postlethwait JH. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 1993, 119: 1203–1215.

12  Ding Y, Xi Y, Chen T, Wang JY, Tao DL, Wu ZL and Li YP,, *et al*.. Caprin-2 enhances canonical Wnt signaling through regulating LRP5/6 phosphorylation. J Cell Biol 2008, 182: 865–872.

13  Rhinn M, Dierich A, Shawlot W, Behringer RR, La Meur M and Ang SL. Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. Development 1998, 125: 845–856.

14  Deschet K, Bourrat F, Ristoratore F, Chourrout D and Joly JS. Expression of the medaka (Oryzias latipes) Ol-Rx3 paired-like gene in two diencephalic derivatives, the eye and the hypthalamus. Mech Dev 1999, 83: 179–182.

15  Lun K and Brand M. A series of no isthmus (noi) alleles of the zebrafish pax2.1 gene reveals multiple signaling events in development of the midbrain-hindbrain boundary. Development 1998, 125: 3049–3062.

16  McClintock JM, Kheirbek MA and Prince VE. Knockdown of duplicated zebrafish hoxb1 genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. Development 2002, 129: 2339–2354.

17  Stachel SE, Grunwald DJ and Myers PZ. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. Development 1993, 117: 1261–1274.

18  Joly JS, Joly C, Schulte-Merker S, Boulekbache H and Condamine H. The ventral and posterior expression of the zebrafish homeobox gene eve1 is perturbed in dorsalized and mutant embryos. Development 1993, 119: 1261–1275.

19  Schulte-Merker S, Ho RK, Herrmann BG and Nusslein-Volhard C. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 1992, 116: 1021–1032.

20  Kishi M, Mizuseki K, Sasai N, Yamazaki H, Shiota K, Nakanishi S and Sasai Y. Requirement of Sox2-mediated signaling for differentiation of early Xenopus neuroectoderm. Development 2000, 127: 791–800.

21  Johnson LR, Lamb KA, Gao Q, Nowling TK and Rizzino A. Role of the transcription factor Sox-2 in the expression of the FGF-4 gene in embryonal carcinoma cells. Mol Reprod Dev 1998, 50: 377–386.

22  Seo HC, Drivenes O, Ellingsen S and Fjose A. Transient expression of a novel Six3-related zebrafish gene during gastrulation and eye formation. Gene 1998, 216: 39–46.

23  Fjose A, Njolstad PR, Nornes S, Molven A and Krauss S. Structure and early embryonic expression of the zebrafish engrailed-2 gene. Mech Dev 1992, 39: 51–62.

24  Munsterberg AE, Kitajewski J, Bumcrot DA, McMahon AP and Lassar AB. Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. Genes Dev 1995, 9: 2911–2922.

25  Ryu SL, Fujii R, Yamanaka Y, Shimizu T, Yabe T, Hirata T and Hibi M, *et al*. Regulation of dharma/bozozok by the Wnt pathway. Dev Biol 2001, 231: 397–409.

26  Gan XQ, Wang JY, Xi Y, Wu ZL, Li YP and Li L. Nuclear Dvl, c-Jun, beta-catenin, and TCF form a complex leading to stabilization of beta-catenin-TCF interaction. J Cell Biol 2008, 180: 1087–1100.

27  Szeto DP and Kimelman D. Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation. Development 2004, 131: 3751–3760.

28  Hashimoto H, Itoh M, Yamanaka Y, Yamashita S, Shimizu T, Solnica-Krezel L and Hibi M, *et al*. Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. Dev Biol 2000, 217: 138–152.

29  Shinya M, Eschbach C, Clark M, Lehrach H and Furutani-Seiki M. Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. Mech Dev 2000, 98: 3–17.

30  Grinblat Y, Gamse J, Patel M and Sive H. Determination of the zebrafish forebrain: induction and patterning. Development 1998, 125: 4403–4416.

31  Kane DA and Kimmel CB. The zebrafish midblastula transition. Development 1993, 119: 447–456.

32  Lekven AC, Thorpe CJ, Waxman JS and Moon RT. Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev Cell 2001, 1: 103–114.

33  Fekany-Lee K, Gonzalez E, Miller-Bertoglio V and Solnica-Krezel L. The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Development 2000, 127: 2333–2345.