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 Bozoizok (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.

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 morphogenetic 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 own 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 were 5'-CGCGGATCCCTT GTAGATTTCCTCATTGCTGCT-3' (sense) and 5'-CGCAATTCCGCCGCTGCCGGAGGACATGCCCAGG-3' (reverse). The whole GFP coding sequence was inserted into NcoI and XbaI 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'-TACATCATGCCCAC TTCTGCTTTGA-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 Drosophila engrailed protein) plasmid, and VP16 chimera construct was generated by inserting the C-terminal deletion fragment into NcoI/ClaI 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 BamHI and EcoRI sites (underlined) of PCS2+ to GFP (green fluorescent protein) plasmid. Primary primer pairs were 5'-CGCGGATCCCTT TGAGATTTCCTCATTGCTGCT-3' (sense) and 5'-CGCAATTCCGCCGCTGCCGGAGGACATGCCCAGG-3' (reverse). The whole GFP coding sequence was inserted into NcoI and XbaI sites of PCS2+ plasmid.
**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 in the neuroectoderm. [Fig. 1(E, E’)] At 10-somites stage, Sox31 distributes along the neural tube, but none transcripts is detected in the optic vesicle. [Fig. 1(F, F’)] At 24 hpf, Sox31 was 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.
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,
its expression domain expanded toward the ventral side in a circular manner, a typical phenomenon of dorsalsalization \[\text{Fig. 3(I–J)}\], while ventral marker eve1 \[18\] completely lost its expression in morphants \[\text{Fig. 3(G,H)}\]. Embryos were fixed at 12 hpf in double and triple staining. Detection of ventral marker eve1 at 50% epiboly in control embryos \(n = 48/55\) \(\text{(H)}\). \(I, I'\) Control embryos show normal expression of gsc at germ ring stage. \(I, I'\) Expression of gsc is significantly expanded towards ventral lateral side in Sox31 morphants \(n = 25/53\). \(K, L\) 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.

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 \(n = 35/48\) \(F\). 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 \[20,21\] \[\text{Fig. 4(A)}\]. 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 dorsalsalization phenotype. The loss-of-function individuals displayed shortened body axis, twisted tail, and protruding telencephalon marked by black triangle \[\text{Fig. 4(B,C)}\]. The brain-midbrain boundary (MHB)
posteriorly shifted from its original position proximal to eye, which was demonstrated by \( \text{pax2a} \) staining [Fig. 4(D,E)]. Thus, \( \text{Sox31} \) 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 \( \text{Sox31} \) 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. \( \text{Sox31} \) overexpression led to significant reduction or absence of the eye, which represented the most anterior neural tissue [Fig. 5(E,G)].

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

Additionally, ectopic \( \text{Sox31} \) led to twisted or shortened notochord with \( \text{ntl} \) expression only detected in the tail bud [Fig. 6(Q–R)]. Somites generation marked by \( \text{myoD} \) [24] was also severely disrupted [Fig. 6(O,P)]. Organizer gene \( \text{gsc} \) was repressed or abrogated from the embryonic shield [Fig. 6(S–T)]. In summary, increase of \( \text{Sox31} \) level results in a dose-dependent loss of anterior head tissue along with

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**Figure 4 Ectopically expression of a DN form of Sox31 phenocopies morpholino effects**

(A) Schematic representation of DN construct for \( \text{Sox31} \), 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 \( \text{pax2a} \) 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.
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. Phentypes 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)].
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 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 ntI 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.
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,I)]. 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
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
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 pre-chordal 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 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.
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.

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