B1 SOX Coordinate Cell Specification with Patterning and Morphogenesis in the Early Zebrafish Embryo

Yuichi Okuda, Eri Ogura, Hisato Kondoh, Yusuke Kamachi*

Graduate School of Frontier Biosciences, Osaka University, Suita, Japan

Abstract
The B1 SOX transcription factors SOX1/2/3/19 have been implicated in various processes of early embryogenesis. However, their regulatory functions in stages from the blastula to early neurula remain largely unknown, primarily because loss-of-function studies have not been informative to date. In our present study, we systematically knocked down the B1 sox genes in zebrafish. Only the quadruple knockdown of the four B1 sox genes sox2/3/19a/19b resulted in very severe developmental abnormalities, confirming that the B1 sox genes are functionally redundant. We characterized the sox2/3/19a/19b quadruple knockdown embryos in detail by examining the changes in gene expression through in situ hybridization, RT–PCR, and microarray analyses. Importantly, these phenotypic analyses revealed that the B1 SOX proteins regulate the following distinct processes: (1) early dorsoventral patterning by controlling bmp2b/b; (2) gastrulation movements via the regulation of pcdh18a/18b and wnt11, a non-canonical Wnt ligand gene; (3) neural differentiation by regulating the Hes-class bHLH gene her3 and the proneural-class bHLH genes neurog1 (positively) and ascl1a (negatively), and regional transcription factor genes, e.g., hesx1, zic1, and nx3; and (4) neural patterning by regulating signaling pathway genes, cyp26a1 in RA signaling, oep in Nodal signaling, shh, and mdkb. Chromatin immunoprecipitation analysis of the her3, hesx1, neurog1, pcdh18a, and cyp26a1 genes further suggests a direct regulation of these genes by B1 SOX. We also found an interesting overlap between the early phenotypes of the B1 sox quadruple knockdown embryos and the maternal-zygotic spg embryos that are devoid of pou5f1 activity. These findings indicate that the B1 SOX proteins control a wide range of developmental regulators in the early embryo through partnering in part with Pou5f1 and possibly with other factors, and suggest that the B1 sox functions are central to coordinating cell fate specification with patterning and morphogenetic processes occurring in the early embryo.

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* E-mail: yusuke@fbs.osaka-u.ac.jp

Introduction
The developing embryo must control gene expression to coordinate various embryonic processes such as cell fate specification, embryo patterning and morphogenesis. During the embryonic stages from the blastula to neurula, the coupling of cell lineage specification and gastrulation cell movements is particularly evident. There is also now an increased understanding of the regulatory mechanisms underlying each cell state and each morphogenetic process, but the precise mechanisms that coordinate these events have remained elusive. The group B1 SOX transcription factors are good candidates as coordinators of these embryonic processes. Indeed, they have been implicated in cell fate specification in the early embryo [1–7] and also patterning and morphogenetic processes [8–10].

B1 SOX comprises sox1a/1b/2/3/19a/19b in zebrafish and Sox1/2/3 in amniotes [11]. The sox19a/19b genes are evolutionary orthologs of mammalian Sox15 (group G), although Sox15 has now been shown to have functionally diversified from the authentic B1 Sox paralogs [11]. Overall, the regulatory functions of B1 sox genes appear to be conserved as a group across vertebrate species, although the paralogs are often differentially employed in a particular process [12]. In zebrafish, sox3/19a/19b are expressed in the blastula [11], whereas the corresponding early expression in mice is covered by Sox2 [1]. Following this stage, the B1 sox genes are thought to be important for specification of the embryonic ectoderm into the neuroectoderm lineage. During this process, their expression becomes confined to the neuroectoderm [11]. As development proceeds to the neurula stage, expression of the B1 sox genes continues in neural precursors, where they function to maintain the neural progenitor states [13–15].

The similarities in the characteristics of the B1 SOX proteins as transcriptional regulators [11,15] suggest redundant functions in tissues where they are coexpressed. In support of this notion, single Sox1 or Sox3 knockout mice display only mild abnormalities in the central nervous system (CNS), presumably because of extensive coexpression of Sox1/2/3 [16–18], whereas Sox2-null mouse embryos die around implantation, reflecting its exclusive expression in the ICM [1]. Consistently, a single sox2 or sox3 knockdown (KD) in zebrafish causes only mild developmental abnormalities [19,20]. Xenopus studies utilizing dominant-negative forms of SOX2 indicate a specific role of Sox2 in neuroectoderm differentiation [2]. To date, however, the overall functions of the B1 sox genes have not been systematically investigated from the blastula to early neurula stages.

An important characteristic of the B1 SOX proteins is that they form a complex with co-DNA-binding partner factors to target
specific sequences and this enables them to participate in the regulation of various cell states [21]. The SOX2-Oct3/4 (Pou5f1) complex is a central player in regulatory networks in the ICM and ES cells [1,22,23]. Potential target genes of SOX2 and Oct3/4 in ES cells have been identified through genome-wide chromatin immunoprecipitation (ChIP) and microarray expression analyses [22,24]. The involvement of other B1 SOX-partner combinations in the regulation of specific cell states has also been reported, e.g., B1 SOX-POUIII factors in neural precursors [15] and B1 SOX-Pax6 in lens cells [25]. However, neither B1 SOX-dependent regulatory processes nor B1 SOX target genes in the developing early embryo have been extensively investigated.

In our present study, we performed single to quadruple knockdowns of sox2/3/19a/19b in zebrafish embryos and confirmed that these four genes are functionally redundant in early development. More importantly, phenotypic analyses of the sox2/3/19a/19b quadruple KD embryos uncovered developmental process-specific functions of B1 sox. In the blastula, B1 sox genes regulate the activation of the bmp2b/7 genes, which is critical for dorsoventral (DV) patterning. During gastrulation, B1 sox also regulate the expression of pcdh18a/18b and wnt11, a non-canonical Wnt ligand gene, which together play a role in convergence and extension (C&E) movements. In neural development, the B1 sox genes are essential for the proper regulation of neural bHLH genes of both the her/Hes and proneural classes, and also for the activation of region-specific transcription factor genes such as hes1, zic1 and vsx3. Moreover, the activity of B1 sox is required for the neural expression of various signaling pathway genes: cyp26a1 in RA signaling, oep in Nodal signaling, shh, and also mdkb. ChIP analysis of the hes2, hes1, neurog1, pcdh18a and cyp26a1 genes suggests their direct regulation by B1 SOX. These findings indicate that B1 SOX proteins play a central role in coordinating cell fate specification, embryo patterning and morphogenesis by controlling a wide variety of developmental regulators in the early embryo.

We have also found an interesting overlap between the early phenotypes of the B1 sox quadruple KD embryos and the maternal-zygotic (MZ) spg embryos that are devoid of pou5f1 activity [26–29]. This highlights a broad role of the B1 SOX-Pou5f1 complex from the blastoderm to early neural stages of development.

Results

Loss-of-function analysis of the B1 sox genes in the zebrafish embryo

Among the B1 sox genes of zebrafish, sox2/3/19a/19b are expressed at high levels during early development with extensive regional overlaps [11]. sox19b mRNA is maternally supplied. sox3 and sox19a are activated around the 1000-cell stage, and sox2 around the 30% epiboly (30%E) stage [11]. The expression of sox2/3/19a/19b initially covers the entire blastoderm, but gradually disappears at the embryonic margin after 30%E (Figure S1A). At the shield stage, the expression of sox2/3/19a/19b covers the future ectoderm, but then becomes confined to the presumptive neuroectoderm [11]. Expression of sox1a/1b is initiated only during late gastrulation stages (Figure S1B). These expression patterns suggest that sox2/3/19a/19b are involved in early processes of zebrafish development.

To investigate the function of B1 sox in early stage embryos, we knocked down sox2/3/19a/19b either individually or in combination using morpholino antisense oligonucleotides (MO). Two different MOs were simultaneously used to block translation of each B1 sox gene, which ensures efficient knockdown even when using reduced amounts of MOs [20]. With this double MO strategy, an approximately 90% reduction in translation was achieved using 1.8 ng of a 1:1 mixture of two MOs, as judged by their effects on luciferase reporters carrying MO-targeting 5’-UTR sequences (sox2 [20]; sox3/19a/19b, Figure S2A). By western blotting, we confirmed the efficient inhibition of the synthesis of endogenous B1 SOX proteins (Figure S2B).

No gross abnormalities were observed in the embryo morphology when any one of sox2/3/19a/19b was knocked down (single KD, Figure 1B), although the development of the CNS may be slightly perturbed and 75% of the sox2 morphants showed an upturned tail phenotype (Figure 1Ba). When any three of sox2/3/19a/19b were simultaneously knocked down (triple KD), a range of morphological abnormalities was observed depending on the combination of KD targets (Figure 1C). Triple KDs of sox2/19a/19b and sox2/3/19b caused only mild morphological defects (Figure 1Cc and 1Cd), presumably because the remaining sox3 and sox19a genes, respectively, mostly cover the B1 sox expression domains. sox3/19a/19b morphants often showed stronger yet variable defects in their posterior structures (Figure 1Ca and 1Cb), presumably reflecting the weak sox2 expression in the posterior neuroectoderm. sox2/3/19a morphants appeared normal during gastrulation, but later developed morphological abnormalities (Figure 1Ce), likely because sox1b expression decreases in later stages.

In contrast to the triple KDs, the quadruple knockdown of sox2/3/19a/19b (hereafter called QKD) using a total of 7.2 ng MOs resulted in very severe developmental abnormalities, suggesting essential functions of B1 sox in early embryogenesis (Figure 1Da–1Df). This result also lends support to a model whereby in the triplet KD embryos the remaining B1 sox gene compensates for the loss of the other three to a large extent. Taken together, our initial observations indicate that the B1 sox genes are largely functionally redundant in early zebrafish embryos. This is further corroborated by rescue experiments to be described in the next section.

By reducing the amount of MOs used for QKD, hypomorphic phenotypes were produced to different extents depending on the MO levels (Figure 1Dg and 1Dh). Embryos injected with a 50%...
Figure 1. Single, triple, and quadruple knockdowns of sox2/3/19a/19b. Bright-field images of live embryos observed at the indicated time points. All are lateral views. (A) Uninjected control (Ctr) embryos. (B) Single knockdowns of sox2/3/19a/19b. A 1:1 mixture of two MOs (0.9 ng each) targeting one of the four B1 sox genes was injected for a single KD. The percentage of embryos in the same morphological class is indicated in each panel. (C) Triple knockdowns of sox2/3/19a/19b. A mixture of indicated combinations of MOs (i.e., a mixture of six MOs, 5.4 ng in total) was used to simultaneously knockdown three out of the four B1 sox genes. The major classes of morphological defects are shown with the percentage of occurrence. The remaining embryos showed either milder or more severe defects. (D) Uninjected control embryos (a–f) and sox2/3/19a/19b quadruple knockdown (QKD) embryos injected with a mixture of MOs targeting the four B1 sox genes (i.e., a mixture of eight MOs, 7.2 ng in total) (a–
The Role of B1 sox in Early Embryogenesis

The QKD caused very severe developmental abnormalities: a delay in epiboly, a shortened anterior-posterior axis, and impairment of CNS development (61%). The remaining embryos showed either milder defects (7%), more severe defects (24%) or lethality (8%). The aberrant movement of the anterior prechordal plate (arrows in d and e') suggests a decreased adhesion of ectodermal cells as well as defects in convergence and extension movements in the QKD embryos. The broken lines (e' and f') indicate the dorsal trunk regions where cell dissociation was observed. (g, h) Dose-dependent effects of the MOs used for QKD were examined by injecting reduced amounts of the mixture of MOs targeting the four B1 sox genes (3.6 ng in total [g] and 1.8 ng in total [h]). (i) As a negative control, a mixture of 5-base-mismatch control MOs (i.e., a mixture of eight 5mis-MOs, 7.2 ng in total) was injected. (j) The coinjection of a p53-MO (2 ng) had no impact on the neural defects in the QKD embryos.

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The earliest detectable morphological abnormality of the QKD embryos was a delay in epiboly, notably after the shield stage (Figure 1D). At 10 hour post-fertilization (hpf), when normal embryos reach the tailbud stage, the QKD embryos were still in late epiboly. The thickening of the anterior head region was less prominent in the QKD embryos (Figure 1Dc and 1Dd), suggesting impairment of CNS development. Impaired CNS development was also indicated by the loss of *hox1* expression in the anterior-most neuroectoderm (Figure 2Bd; see also Figure 3Ca) and by the anterolateral displacement of the *pax2a* expression domains that mark the midbrain-hindbrain boundary (MHB) (Figure 2Bd). An early phase of neurogenesis was also affected in the QKD embryos as indicated by the loss of proneural *neurog1* expression (Figure 2Bf). The QKD embryos further displayed a shortened anterior-posterior (AP) axis with a broadened neural plate (marked by *hoxb1b*) and broadened mesodermal structures including notochord (marked by *ntf*) (Figure 1D and Figure 2B). Consistently, the gap between the prechordal plate (marked by *hgg1*) and notochord was reduced in the QKD embryos (Figure 2Be). These abnormalities commonly occur in zebrafish embryos when C&E movements are impaired during gastrulation [31,32].

**Interchangeable B1 SOX protein functions during early zebrafish development**

The effects of the B1 sox KDs suggested that the B1 SOX proteins act equivalently in transcriptional regulation in the zebrafish embryo. Indeed, SOX1/2/3/19 all activate the *nestin* and *-crystallin* enhancers in cooperation with Brn2 and Pax6, respectively, in cultured cells [11]. We therefore tested whether injection of a single B1 sox mRNA could rescue the QKD phenotype. Moderate amounts (20–30 pg) of B1 sox mRNAs lacking the MO-target 5′ UTR sequences were individually injected with the MOs for QKD. Coinjection with any one of the *sox2*/*3*/*19a*/*19b* mRNAs dramatically rescued the QKD phenotype, as judged by the recovery of a normal morphology (Figure 2Ca–2Cf). By measuring the AP axis length of the embryos at 15-16 hpf, we confirmed the recovery of axial elongation in the B1 sox mRNA-injected QKD embryos (Figure 2D). In these embryos also, the expression of *hox1, ddx3b* (neural plate border) and *neurog1* was recovered and the expression patterns of *pax2a* and *hoxb1b* were restored (Figure 2Cd–2Cf). In addition, the C&E movements indicated by the expression patterns of *hgg1, ntl* and *ddx3b* [31] were normalized in the B1 sox mRNA-injected embryos (Figure 2Ce). This phenotypic rescue was efficient only to early somitogenesis stages, likely because of the gradual decrease in the exogenously supplied SOX expression [19]. Simultaneous injection of *sox2/3/*19a/*19b* mRNAs (5 pg each) had essentially the same rescue effects (data not shown). These observations indicate that the function of the B1 SOX proteins is interchangeable during early zebrafish development.

**Phenotypes of B1 sox-deficient embryos**

To further explore the functions of B1 sox in early zebrafish embryogenesis, we characterized our QKD embryos by focusing on their defects in neural development and gastrulation movements. In these QKD embryos, expression domains of *otx2* (anterior neuroectoderm) and *zic2b* (entire neuroectoderm) were expanded ventrally at 75%, whereas expression of both *fini* and *ddx3b* (non-neuroectoderm) was reduced (Figure 3Aa–3Ad). Consistently, the neuroectodermal expression of B1 sox was also expanded in the QKD embryo (Figure 3Ae–3Ah). These gene expression changes are reminiscent of the dorsalized phenotype seen in BMP-pathway mutants [33,34], suggesting impairment of this pathway in the QKD embryos.

At the tailbud stage in the QKD embryos, expression domains of *pax2a, gbx1* (hindbrain) and *hoxb1b* (posterior neuroectoderm) were anterolaterally shifted, which encompass the *otx2* expression domain (Figure 2Bd and Figure 3B). A similar change was seen for *eng2a* expression in the MHB at the 3-somite stage (Figure 3Bf). These observations indicate that some characteristics of the early neural plate can develop even under a severe reduction of B1 SOX activity. However, our initial analyses revealed that expression of many neural genes is abolished in the QKD embryos, including *hesx1*, *krox20* (rhombomere r) [3/5] as well as *hex1* and *neurog1* (Figure 2B and Figure 3C). Injection of the MOs for QKD into embryos of the *nr2f2* enhancer-trap line [35] confirmed the impairment of brain development at later stages (Figure S4A). These findings together suggest that B1 sox activity is critical for neural development, although it is dispensable for the expression of some early neural genes.

The normal expression levels of *ntl* and *myod1* (somite) in the QKD embryos suggest that mesodermal differentiation per se can proceed (Figure 3D). However, the broadened expression domains of these genes indicate defects in convergence movements, which is consistent with the widened expression of neural genes such as *gbx1* and *hoxb1b*. Movement of the anterior prechordal plate (marked by *hgg1*) was also impaired in the QKD embryos (Figure 2Be), which is characteristic of defective C&E movements. However, hatching gland precursor cells were commonly found to aberrantly move in a dorsal direction and penetrated the ectoderm during mid-somite stages (Figure 1De'). In the severe morphants, these hatching gland cells remained as a single ball-like structure in the head (Figure S1B). This phenotype is unique to the QKD embryo and may reflect a decreased adhesion of ectodermal cells, as also suggested by cell dissociation from the dorsal trunk region (Figure 1De' and 1Df).
Figure 2. Rescue of the QKD phenotype by exogenous B1 sox mRNAs. (A, B) Uninjected control (Ctr) embryos (A) and the QKD embryos (B). Live embryos were observed at 10–11 (a), 15–16 (b) and 30–31 (c) hpf. Expression of hesx1, pax2a and hoxb1b (d), dlx3b, hgg1 and ntl (e), and neurog1 (f) was visualized by whole-mount in situ hybridization. Lateral views (a–c); dorsal views with anterior to the top (d–f). (C) The QKD phenotype is similarly rescued by an exogenous supply of any B1 sox mRNA. The MOs for QKD were coinjected with the indicated mRNAs. In the B1 sox mRNA-coinjected embryos, the expression of hesx1, dlx3b and neurog1 was recovered; patterning of the neural plate marked by pax2a and hoxb1b was normalized; and the expression patterns of hgg1, ntl and dlx3b reflecting C&E movements were also restored. Blue bracket, gap between the hgg1 and ntl expression domains; white dotted line, neural plate border. (D) Recovery of the AP axis elongation in QKD embryos injected with one of the B1 sox mRNAs. The length of the embryos at 15–16 hpf along the AP axis between the arrowheads (Ab, Bb, Cb) was measured for the uninjected control (Ctr) (n = 7), the QKD (n = 9) and QKD with B1 sox mRNA injection (sox2, n = 9; sox3, n = 6; sox19a, n = 7; sox19b, n = 6). The average AP axis lengths with standard errors are shown.

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Figure 3. Defects in embryo patterning, early neural development, and C&E movements in the QKD embryos. Comparison of the gene expression profiles between uninjected control (Ctr) and QKD embryos. (A) Ventral expansion of early neural gene expression (otx2 [a], zic2b [b] and sox2/3/19a/19b [c–h]), and reduced expression of non-neural ectoderm marker genes (foxi1 [c] and dlx3b [d]) at 75%E. (B) Altered expression patterns of neural genes at the tailbud and 3-somite stages. The expression domains of gbx1 (c–e), eng2a (f) and hoxb1b (g, h) were anterolaterally shifted, which encompass the otx2 expression domain (a, b). (C) Defective neural development revealed by the loss of expression of neural marker genes,
Interestingly, the QKD embryos show an increase in transcript levels of \textit{sox2/3/19a/19b} and also \textit{sox1b} at early developmental stages (Figure 3A and Figure S1B). This implies a negative autoregulation of transcription among the B1 \textit{sox} members, although a stabilization of these mRNAs by the MOs could not be ruled out. It is also noteworthy that even with these elevated levels of B1 \textit{sox} transcripts in the QKD embryos, the MOf-mediated knockdowns were effective in inhibiting their translation as revealed by western blotting (Figure S2B).

To further characterize the phenotype of the QKD embryos, we examined the changes in gene expression in greater detail by the combined use of in situ hybridization, RT-PCR (summarized in Table S1) and microarray analysis (Figure S3, Table S2 and Table S3). Overall, these analyses indicated that a wide range of developmental processes were affected in the QKD embryos and the major phenotypes can be categorized into: (1) the early dorsoventral patterning defects; (2) defects in gastrulation movements; (3) dysregulation of early neural and neuronal regulatory genes; (4) neural patterning defects associated with the abrogated expression of signaling pathway genes; and (5) early defects resembling those observed for MZ\textit{pg} embryos. Further details of these phenotypes are described below.

**Aberrant DV patterning in B1 \textit{sox} QKD embryos caused by dysregulation of \textit{bmp} genes**

Early DV patterning of the embryo relies on a gradient of Bmp signaling, in which \textit{bmp2b/7} play a major role in zebrafish [36,37]. The phenotypic similarities between the QKD embryos and \textit{bmp} pathway mutants described above prompted us to examine the genes in this pathway. Expression of \textit{bmp2b/7} was found to be reduced in the QKD embryos from the beginning (Figure 4Aa–4Af). \textit{bmp4} expression levels were more or less normal initially, but were downregulated at late epiboly stages in the QKD embryos (Figure 4Ag; data not shown). Consistently, expression of the \textit{gata2}, \textit{szl} and \textit{eve1} genes, which are immediately downstream of Bmp signaling, was also reduced in the QKD embryos (Figure 4Ah–4Aj).

The dorsal identity of the zebrafish embryo requires activation of maternal \textit{β}-catenin, which then activates expression of \textit{gsc} and the Bmp antagonist genes \textit{chd} and \textit{nog1} at the dorsal side. In the QKD embryos, these genes were initiated normally, although the expression of \textit{chd} was slightly ventrally expanded at 30%E (Figure 4Ak), which is likely secondary to the reduced expression of \textit{bmp2b/7}. At later stages in the QKD embryos, however, \textit{chd} expression was rather decreased (Figure 4Aam), contrasting to the \textit{bmp} pathway mutants [38].

To determine the relationship between the dorsoalyzed phenotype of the QKD embryos and Bmp signaling, we injected a mixture of \textit{bmp2b/7} mRNAs (20 or 40 pg each) together with the MOs for QKD. This \textit{bmp2b/7} injection rescued the expression of the Bnp downstream genes \textit{gata2}, \textit{szl} and \textit{eve1} (Figure 4B), indicating that signaling components acting downstream of \textit{bmp2b/7} are not affected in the QKD embryos. Consistently, the mRNA levels of Bmp receptor genes \textit{(acvrl1, bmp1aa, bmp1ab} and \textit{bmp1ba}) and \textit{smad5} were found to be normal in the QKD embryos by microarray (GEO accession number GSE18830). These observations, together with the normal initiation of the dorsal pathway in the QKD embryos, indicate that the DV patterning defects of the QKD embryos primarily result from the reduction of \textit{bmp2b/7} expression.

**B1 \textit{sox} are required for proper gastrulation movements**

Components of non-canonical Wnt signaling and cell adhesion molecules are major regulators of gastrulation movements, including epiboly and C&E movements [32,39]. Since these movements are severely impaired in the QKD embryos, we investigated expression profiles of genes related to these processes. \textit{wnt11} and \textit{wnt5b} are major Wnt ligand genes involved in C&E movements [31,32]. In the QKD embryos, upregulation of \textit{wnt11} that normally occurs during early gastrulation was not observed and expression of \textit{wnt5b} was slightly reduced at late epiboly stages (Figure 4C). We also observed decreased expression of \textit{wnt11} and \textit{wnt5a}, which are important for convergence movements at later stages [40]. These data suggest that the reduction of non-canonical Wnt ligands contributes to the impairment of C&E movements.

Both classical cadherins and protocadherins are involved in gastrulation movements [39]. We found that expression of \textit{pdcbl1bα/18b} was significantly reduced in the QKD embryos (Figure 4C). These genes are expressed in the epiblast at the shield stage and later in the neuroectoderm in an overlapping manner in normal embryos [41,42]. To examine how reduced activity of \textit{Pdcbl1bα} affects embryogenesis, we knocked down these two genes. Although only mild gastrulation defects were observed when these genes were knocked down separately, simultaneous KD caused a delay in epiboly and also C&E defects (Figure S6; for \textit{pdcbl1bα single KD}, see also [41]). Delayed epiboly has also been reported for the hypomorphic \textit{cdk1} mutants [43,44], but its expression was not altered in our QKD embryos (Figure 4C). These observations indicate that multiple mechanisms involved in gastrulation movements are simultaneously affected in the QKD embryos.

**Dependence of neuronal differentiation programs on B1 \textit{sox} activity**

Several neuronal genes were found to be abnormally upregulated in the QKD embryos. \textit{smnt2a} is strongly expressed in CNS neurons from mid-somitogenesis stages in wild-type embryos [45] and also weakly expressed throughout the embryo at epiboly stages (Figure 5A and Figure S7A). The latter early stage expression was found to be aberrantly upregulated in the QKD embryos (Figure 5A and Figure S7A). The neuronal \textit{tubal1} gene was also upregulated from 75%E (Figure 5A). These observations suggest that a portion of the neuronal differentiation programs is precociously initiated in the QKD embryos.

Neural bHLH transcription factors are key players in the neuronal differentiation programs. \textit{Hes/Her} genes encode repressors of bHLH proteins, are expressed in undifferentiated neural progenitor cells and maintain their cell state [46]. Among the zebrafish \textit{her} genes, \textit{her3}, an ortholog of mammalian \textit{Hes3}, is initiated in the dorsal region of the epiblast at about 30%E, and its expression continues in bilateral inter-proneuronal domains [47]. This \textit{her3} expression is totally lost in the QKD embryos (Figure 5A and 5Ba–5Bc).

Proenural genes encoding activator-type bHLH proteins and participating in neurogenesis are also affected in the QKD embryos. In normal zebrafish embryos, \textit{neurog1} expression initially marks primary neurons at the end of gastrulation and then covers the proneural domains in a fashion complementary to \textit{her3} expression. In the QKD embryos, \textit{neurog1} expression is also lost (Figure 2Bi and Figure 5A). However, not all proenural genes behave in this manner as for example \textit{ascl1a} is transiently...
Figure 4. Defects in DV patterning and gastrulation movements in the QKD embryos. (A) DV patterning defects involving the reduced expression of bmp genes. Expression of bmp2b/7/4 (a–g) and the Bmp downstream genes gata2, szl and eve1 (h–j) is reduced in the QKD embryos. (k–s) Expression of the Bmp antagonist genes, chd and nog1, and the organizer gene gsc. All are lateral views with dorsal to the right. (B) The BMP downstream genes are restored by an exogenous supply of bmp2b/7. Embryos were injected together with the MOs for QKD and a mixture of bmp2b/7 mRNAs (20 or 40 pg each) and subjected to RT-PCR analysis at the shield stage. bactin1 was used as an RT-PCR control. (C) Decreased expression of genes regulating C&E movements in the QKD embryos. Temporal expression profiles of the indicated genes in the uninjected control and QKD embryos from the sphere to 3-somite stages were determined by RT-PCR. Expression of non-canonical wnt genes is reduced in the QKD embryos. Expression of pcdh18a/18b is also reduced in the QKD embryos, whereas cdh1, which is known to be involved in epiboly, is expressed at normal levels. bactin1 was used as an RT-PCR control.

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upregulated at about 75% in a broad area of the neuroectoderm in the QKD embryos (Figure 5A and 5B). Proneural genes are known to be repressed by Hes/Her [46], but exogenous injection of her3 mRNA into QKD embryos did not repress astral expression (data not shown), indicating that the loss of her3 was not causal to this upregulation. The neuronal repressor REST has been implicated in suppression of astral as well as Stmn2 [48]. However, REST expression was unchanged in the QKD embryos (Figure 5A), although REST is suggested to be downstream of SOX2 in ES cells [24], indicating that REST is not involved in the aberrant regulation of astral and Stmn2.

Taken together, our results indicate that the proper operation of the neuronal differentiation programs, including regulatory networks involving the neural bHLH genes, is highly dependent on the activity of B1 sox.

Involvement of B1 sox functions in neural patterning
Regional identities of the neural plate are specified through regulatory networks involving various signaling pathways and transcriptional regulators. Genes that are critical for these networks are severely affected in the QKD embryos. As described earlier, in the QKD embryos, expression of the transcription factor genes her, zic1, and rx3, which are required for forebrain and eye development [49–51], is lost throughout early embryogenesis (Figure 3C and Figure 5A). The MHB itself was established, as judged from the expression of px2a and eng2a, but the anterolaterally-shifted expression patterns of these genes suggest an improper formation of the axes of the anterior neural plate (Figure 2B and Figure 3B). The expression domain of otx2 was expanded and encircled by those of px2a, eng2a and gbx1 (Figure 2B and Figure 3B), which is likely due to the dorsalized phenotype caused by the decreased expression of bmp2b/7, as the bmp2b/7 mutant embryos show similar patterns of gene expression [33,34]. In addition, the QKD embryos lacked the anterior neuroectoderm expression of cyp26a1 (Figure 5C), which encodes an RA degrading enzyme and thereby plays a role in hindbrain patterning [52]. Hence, the reduction of cyp26a1 expression partly accounts for the hindbrain defects in the QKD embryos, e.g., expansion of her7 in r4 (Figure 5C) as observed in the cyp26a1 mutant [53]. However, B1 sox also seem to be more directly involved in gene regulation in hindbrain development as evidenced by severe downregulation of astl in r5/6 (Figure 5Cd) and the loss of krox20 expression in r3/5 (Figure 5Ce).

Nodal and Sonic hedgehog signaling are crucial for the development of ventral brain structures [54–56]. In normal embryos, oep, an ortholog of mouse Cripto, is strongly expressed in the anterior neural plate and is essential as a coreceptor for receiving Nodal signals [54]. Interestingly, oep expression in the ectoderm at the shield stage and the neuroectoderm at later stages is selectively lost in the QKD embryos (Figure 5Db–5Dd), whereas its early zygotic and mesodermal expression was maintained (Figure 5Da–5Dc). Moreover, expression of shha and shhb in the ventral floor of the brain is also lost in the QKD embryos, leaving only shhb expression in the prechordal plate (Figure 5De–5Dd). These findings suggest that the loss of oep and shhb expression leads to defective ventral brain development in the QKD embryos. It is known that shha expression in the neuroectoderm is regulated by Nodal signals from the mesoderm [57]. However, a more direct link between B1 SOX action and shhb regulation is suggested, as the exogenous injection of oep mRNA into the QKD embryos did not restore the shha expression (data not shown), although Nodal-encoding ndr2 is normally expressed (Figure 5De).

Defects in the anterior neural plate development in the QKD embryos also include the loss of mdkb expression (Figure 5E). Consistent with the proposed role of mdkb in the specification of neural crest cells [58], mdkb expression in the neural crest was reduced in the QKD embryos (Figure 5Ec).

Regulatory actions of the B1 SOX proteins in early zebrafish embryos
As the B1 SOX proteins primarily function as transcriptional activators [11,13,15,23,25], the expression of direct target genes is expected to be decreased in response to the QKD. However, the upregulation of several neuronal genes such as stmn2 raised the possibility that B1 SOX might also act as repressors. To test this, we utilized dominant activator and repressor forms of SOX3, SOX3-VP16 and SOX3-EnR (Figure 6A) and compared the effects of these variants under QKD conditions with those of SOX3. As anticipated, genes that were downregulated in the QKD embryos, namely bmp2b/7, pdxh1a/b, her3, her1 and zic1, were efficiently recovered by the exogenous supply of either SOX3 or SOX3-VP16 but not by SOX3-EnR (Figure 6B). These genes are thus likely activation targets of B1 SOX. In addition, the increased expression of stmn2a and astral in the QKD embryos was suppressed in the same way. This suggests an indirect regulation of these genes by B1 SOX through the activation of repressors. However, SOX3-VP16 was less effective than SOX3 in the rescue of some genes such as pdxh1a/b and astral, suggesting that the activation process may require additional molecular interactions with the intact SOX3 protein.

It is noteworthy that the morphological rescue of the QKD embryos by SOX3-VP16 was much less complete when compared to that observed for SOX3, and that the SOX3-VP16-injected embryos showed a rather ventralized phenotype (Figure 6C). In line with this observation, chd and nog1 were unexpectedly reduced in SOX3-VP16-injected embryos, but increased in SOX3-EnR-injected embryos, suggesting that the repressive action of B1 SOX may be required for the proper regulation of dorsally expressed BMP antagonist genes. These findings together indicate that the B1 SOX proteins primarily act as activators in early embryos, whereas a context-dependent repressive action of these factors is also suggested.

Direct regulatory targets of B1 SOX in early zebrafish embryos
To further explore whether the B1 SOX proteins directly regulate the potential downstream genes described above, we
searched for possible B1 SOX binding sites (containing the consensus sequence CATTGTT [21,59] or closely related sequences) in the regulatory regions of these genes. We identified potential SOX-binding sites in the regulatory sequences of her3 [47], hesx1, cyp26a1 [60] and neurog1 [61] and also in the conserved non-coding sequences upstream of pcdh18a (Figure 7Aa). To investigate the direct interaction of the B1 SOX proteins with these genomic sequences in vivo, ChIP experiments were performed using zebrafish embryos at the 70–80% E and tailbud to 2-somite stages. ChIP analysis using anti-SOX2 antibody that weakly cross-reacts with SOX3-VP16 and SOX3-EnR (20 pg) were individually injected with the MOs for QKD and gene expression responses were examined by RT-PCR. The exogenous supply of either SOX3 or SOX3-VP16 but not by SOX3-EnR recovered expression of genes that were downregulated (bmp2b, pcdh18a/18b, her3, hesx1 and zic1) in the QKD embryos and also suppressed expression of genes that were upregulated (stmn2a and asc1a). bactin1 was used as a RT-PCR control. These data confirm

To further investigate whether the activities of these regulatory sequences are dependent on B1 SOX, we created luciferase reporter vectors containing the promoter sequences for hesx1 and cyp26a1 [60] (Figure 7Ba). The 0.8-kb promoter sequence of the zebrafish hesx1 gene used here corresponds to the chicken Hesx1 promoter that has been shown to have anterior CNS-specific regulatory activity in chicken and also zebrafish embryos [62]. The conserved non-coding sequence upstream of pcdh18a (412 bp) was also cloned into the TK-luciferase reporter vector (Figure 7Ba). When these reporter vectors were injected with or without the MOs for QKD into zebrafish embryos, the promoter activities of hesx1 and cyp26a1 were significantly downregulated upon B1 sox QKD (Figure 7Bb). The 412-bp pcdh18a sequence showed an enhancer activity in normal embryos, whereas this activity was also reduced in the QKD embryos (Figure 7Bb). These data confirm
Figure 7. Direct regulatory targets of the B1 SOX proteins in the zebrafish embryo. (A) ChIP analysis showing direct association of B1 SOX with regulatory sequences of the downstream genes. (a) Potential binding sites for SOX and POU within the analyzed genomic regions are schematically shown. (b) ChIP-PCR analysis using anti-SOX2 antibody. ChIP experiments were performed using zebrafish embryos at the 70–80%E and tailbud to 2-somite stages. ChIP-PCR analysis using anti-SOX2 antibody that weakly cross-reacts with SOX3/19A/19B revealed specific binding of B1 SOX to the regulatory sequences of the \textit{hesx1}, \textit{her3}, \textit{pcdh18a}, \textit{cyp26a1} and \textit{neurog1} genes in the zebrafish embryo. \textit{bactin2} was used as a negative control. (B) B1 SOX-dependent activities of the regulatory sequences of \textit{hesx1}, \textit{cyp26a1} and \textit{pcdh18a}. (a) The Venus luciferase (Venus plus firefly luciferase) constructs containing either of the promoters for \textit{hesx1} or \textit{cyp26a1} or the upstream conserved sequence of \textit{pcdh18a} with the HSV TK promoter are schematically shown. (b) The Venus luciferase reporters were injected into embryos with or without the MOs for QKD together with the reference vector TK-Renilla luciferase. More than 20 injected embryos per sample were collected at the tailbud stage, and luciferase assays were performed. The normalized luciferase activity generated by TK-Venusluc was arbitrarily assigned a value of 1. Data are shown as the average values of four independent injection experiments with standard errors.

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that B1 SOX proteins regulate the hesx1, cyp26a1 and pcdh18a genes through these regulatory elements.

Interestingly, POU binding sites were found abutting the SOX sites of her3 and hesx1. These genes were found to be commonly downregulated in the QKD embryos (Figure 3C and Figure 5A and 5B) and also MZspg mutants (Table S7; see also [29]). In the QKD embryos, Pou5f1 is expressed at normal levels (Figure S7B), indicating that Pou5f1 alone is insufficient to induce her3 or hesx1. These data together suggest that B1 SOX and Pou5f1 proteins synergistically cooperate to activate her3 and hesx1.

Discussion

Previous studies have suggested that the group B1 sox genes are critical for early processes in embryogenesis, particularly during early neural development [1–6]. Possibly as a consequence of functional redundancy, however, loss-of-function analyses have not been sufficiently informative to date. In our present study, we successfully depleted the B1 sox activity from early zebrafish embryos by a quadruple knockdown of sox2/3/19a/19b, and present clear evidence that the B1 sox genes are highly redundant and their encoding proteins are functionally interchangeable in early zebrafish embryogenesis. More importantly, we demonstrate that the B1 sox genes are indeed essential for several key processes during early embryogenesis, namely embryonic patterning, gastrulation movements and neural development. The major downstream genes of B1 SOX that function in these processes were found to be developmental transcription factor genes, signaling pathway genes and cell adhesion molecule genes (Figure 8). These data indicate that B1 SOX proteins play a central role in coordinating cell fate specification with embryo patterning and morphogenetic processes by controlling a wide
variety of developmental regulators in a process-dependent manner. Among the broad functions of B1 SOX, the transcriptional partnership with Pou5f1 is critical for early embryogenesis from the blastoderm to early neural stages as detailed further below.

The partnership of B1 SOX and Pou5f1, a homolog of mammalian Oct3/4, in early embryogenesis

We found similarities in the gene expression profiles between the B1 sox QKD embryos and the MZ spg embryos in a wider range of developmental stages from the blastoderm to early neural stages (Figure 8, Table S7 and [29]). This strongly suggests that their cooperation is required not only for the blastoderm stage, which may be similar to the E5 cell state, but also for the early neural stage. In contrast to mouse knockouts of Sox2 or Oct3/4, where impairment of the ICM/epiblast lineage development causes early embryonic lethality [1,63], zebrafish embryos of the B1 sox QKD and MZ spg mutants are viable although with severe developmental defects. This enabled us to analyze the functions of B1 sox in later developmental stages as well as the blastoderm stage.

A group of key genes downstream of B1 SOX and Pou5f1 in the blastoderm were found to be bmp2b/7, as the expression of bmp2b/7 is also severely reduced in MZ spg embryos [28]. In addition, an overlap of the expression domains of B1 sox, pou5f1 and bmp2b/7 in the blastoderm strongly suggests a direct regulation of bmp2b/7 by B1 SOX and Pou5f1. The dorsalized phenotype of the B1 sox QKD and MZ spg embryos can to a large extent be ascribed to the bmp2b/7 defects, as bmp2b/7 mRNA injection into the QKD embryos rescues the expression of Bmp downstream genes (Figure 4B) and bmp2b mRNA injection can also rescue the MZ spg embryos [28]. This co-regulation thus appears to be critical for the establishment of the early DV axis, but likely operates only during the initial activation of bmp genes, since during gastrulation the expression domains of B1 sox and bmp2b/7 segregate and eventually become complementary to each other.

her3 and hesx1, which both encode transcriptional repressors, were identified as direct targets of B1 SOX in the early phase of neural development (Figure 5, Figure 6, and Figure 7). The expression of these genes is also lost in the MZ spg embryos (Table S7 and [29]). In addition, our ChIP analysis indicated that the proximal and distal SOX-POU elements of the her3 promoter and the hesx1 promoter carrying multiple SOX and POU sites are bound by B1 SOX in vivo (Figure 7A). We further verified, using a luciferase reporter assay, that the activity of hesx1 promoter is dependent upon B1 SOX. These observations together indicate that her3 and hesx1 are regulated under the cooperative action of B1 SOX and Pou5f1. In addition, the B1 SOX and Pou5f1 complex appears to be required for expression of hypothetical repressors that inhibit neuronal differentiation, since the expression of stem2a is aberrantly upregulated in the B1 sox QKD embryos and also in MZ spg embryos (Figure 5A, Figure S7A, and Table S7). These data indicate that in the early phases of neural development B1 SOX proteins cooperate with Pou5f1 and activate the transcriptional repressors genes that inhibit the further differentiation of neural progenitor cells.

The role of the B1 sox genes in coordinating the developmental processes in the gastrulating embryo

In the gastrulating embryo, cell fate specification must be coupled with embryo patterning and gastrulation movements. We found that B1 SOX proteins are involved in all these processes by controlling their respective regulators in the gastrulating embryo.

In the early phase of neural fate specification, the B1 SOX proteins are required for the activation of her3 and the repression of ascl1a, which by themselves are inhibitory to neuronal differentiation (Figure 8). On the other hand, B1 SOX also appear to play a role in an initial phase of neuronal differentiation by directly activating the proneural neurog1 gene. neurog1 is severely downregulated in the QKD embryos (Figure 2 and Figure 5), and in vivo binding of B1 SOX to its regulatory sequence LSE is indicated by our ChIP analysis. In addition, although B1 SOX proteins are known to counteract neurogenesis, this inhibition occurs at late steps of neurogenesis without affecting Neurog1/2 expression [13]. Taken together, these data indicate that B1 SOX are important for the successive generation of neural progenitor cells and immature neuronal cells.

Another important aspect of the functions of the B1 sox genes during neural lineage differentiation is that the initiation of the transcription factor genes zic1 and rx3 depends on their activity (Figure 3 and Figure 5). rx3 may also be directly regulated by B1 SOX, since Xenopus Rx1, a functional homolog of zebrafish rx3, is under the direct regulation of SOX2 and Otx2 in the eye field [64]. Zic1 and Rx3 generally act as a transcriptional activator and are required for forebrain and eye lineage development [50,51].

Critical functions of B1 sox in embryo patterning are underscored by our present findings that the neural expression of the signaling pathway genes cyp26a1, oep, shha/b and mdkb is dependent upon B1 sox activity (Figure 5). Signaling pathways involving these genes play a key role in cell fate decisions as well as diverse patterning processes in the developing CNS [52–56,58]. Our ChIP and promoter analyses suggest a direct regulation of cyp26a1 by SOX1 (Figure 7). Furthermore, the expression of oep, mdkb and shha/b extensively overlaps with that of B1 sox in the neuroectoderm, also implying direct regulation by B1 SOX. Interestingly, the expression of Shh during mouse hippocampal development has recently been shown to be directly regulated by SOX2 [65].

A remarkable defect of the QKD embryos was also found to occur in gastrulation movements. Delayed epiboly and impaired C&E movements are also shared phenotypes with the MZ spg embryos [26–28], suggesting that these processes may also be co-regulated by B1 SOX and Pou5f1. We speculate that a severe reduction of pcdh18a/18b in combination with a reduced expression of non-canonical wnt genes is largely responsible for the defects in epiboly and C&E movements of the QKD embryos (Figure 4). We have further shown in our present analyses that the conserved sequence block upstream of pcdh18a acts as a B1 SOX-dependent enhancer. The in vivo binding of B1 SOX to this enhancer indicated by our ChIP analysis further supports a direct regulation of pcdh18a by B1 SOX. The knockdown phenotypes of pcdh18a/18b were consistent with their important functions in gastrulation movements (Figure S6 and [41]), but their molecular role in this process is still unclear. Recent studies, however, have reinforced the critical role of cell adhesion molecules in gastrulation movements [39]. Pcdh8 (Papc), structurally similar to Pcdh18, controls C&E movements of the paraxial mesoderm in cooperation with the non-canonical Wnt pathway [66], suggesting analogous roles of Pcdh18a/18b in the ectoderm.

The findings of our current study thus demonstrate that the B1 SOX proteins regulate genes that are critical for a variety of processes in early embryonic development. This suggests that these factors serve as central coordinators of gene regulatory networks in the early developing embryo by coupling cell fate specification with patterning and morphogenetic processes. In transcriptional regulation, B1 SOX proteins likely perform this coordination by partnering with a variety of factors including Pou5f1 [21].

The Role of B1 sox in Early Embryogenesis

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microarray analysis also suggests that B1 SOX regulate additional genes and pathways that we did not investigate herein. Future studies of these genes will therefore more fully delineate their multiple functions in coordinating early embryogenesis.

Materials and Methods

MO-mediated knockdowns

MOs were obtained from Gene Tools LLC (OR, USA) and are listed in Table S4. Zebrafish embryos were obtained by natural matings of wild-type TL fish and reared at 28.5°C in 0.03% Red Sea salt solution. Approximately 1 nl of solution containing various combinations of MOs, as indicated in the figures, was injected into 1-cell stage embryos. Unless otherwise noted, a 1:1 mixture of two MOs (0.9 ng/ml each) was used to knockdown individual B1 sox genes. To knockdown multiple B1 sox genes, the MOs were each mixed at a concentration of 0.9 ng/ml and injected into 1-cell stage embryos.

To estimate the knockdown efficiency of B1 sox MOs, fusion mRNAs of sox3-luc, sox19a-luc and sox19b-luc were prepared by transcription of template vectors, in which the 5′-UTR sequence and a short stretch of the amino-terminal-coding-sequence of the respective genes (−131 to +32 of sox3; −151 to +52 of sox19a; −151 to +32 of sox19b) were inserted upstream of the luciferase sequence as previously described [20]. mRNA were microinjected with the relevant MOs into embryos and luciferase activities expressed in the embryos at 10–11 hpf were measured as described [20].

Gene expression analysis

Whole-mount in situ hybridizations of zebrafish embryos were performed as described previously [11]. The genes analyzed are listed in Table S1.

Total RNAs for RT-PCR and microarray analyses were prepared from 40–100 uninjected control embryos and an equivalent number of embryos injected with MOs and/or mRNAs using RiboPure kit (Ambion, TX, USA). 200 ng of total RNA of each sample was reverse-transcribed using oligo-dT primer and Superscript III RT-PCR system (Invitrogen, CA, USA) and a 1/80 fraction of the cDNA was used for PCR templates. PCR was performed using ExTaq polymerase (Takara, Japan) in 25 µl ExTaq buffer containing 5% dimethyl sulfoxide, 0.17 mM crenosol red, 10% sucrose and primers listed in Table S3. The PCR temperature profile consisted of 5 min denaturation, 23–30 cycles of a 30 sec denaturation at 94°C, 30 sec annealing at 57–61°C and 15 sec primer extension at 72°C and lastly a 10 min extension at 72°C (see Table S3 for cycle numbers and annealing temperatures). PCR products were separated in a 2% agarose gel (1.5% Methaphor agarose/0.5% agarose) and stained with SYBR green I.

For microarray analysis, cRNA probes were prepared using 4 µg total RNA with a one-cycle cDNA synthesis kit (Affymetrix, CA, USA). Affymetrix Zebrafish Genome arrays were hybridized with 10 µg cRNA probes, and posthybridization staining and washing were performed according to the manufacturer’s instructions. RNAs from two independent samples were analyzed for each embryonic stage and the data were processed using the RMA program. Fold changes of the averaged hybridization signals between control and QKD embryo samples were then determined (Figure S5, Table S2 and Table S3). The microarray data have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) at the National Center for Biotechnology Information with the accession number GSE18830.

Western blotting

Western blotting with an anti-SOX2 antibody was carried out as described previously [20]. For the detection of SOX3, SOX19A and SOX19B, an anti-SOX3 C-terminal peptide antibody [15] was used. As a loading control, the blotted PVDF membranes were stained with Coomassie Brilliant Blue R-250.

Synthetic mRNAs for embryo injection

The coding sequences of the B1 sox genes and their derivatives were cloned into the pCBA3 vector [20]. The coding sequence of bmp7 was amplified by RT-PCR and cloned into pCBA3. The cDNA clone cb670 (Zebrafish International Resource Center [ZIRC], OR, USA) was used as a template for bmp2b mRNA. mRNAs were transcribed in vitro from linearized vectors using the mMessage mMachin SP6 kit (B1 sox and bmp7) or mMessage mMachin T7 Ultra kit (bmp2b) (Ambion). For the rescue experiments, each mRNA was mixed with the MOs for QKD and injected into 1-cell stage embryos.

Chromatin immunoprecipitation

ChIP was carried out as described previously [67] with minor modifications. Briefly, zebrafish embryos at the 70–80% E and tailbud to 2-somite stages were enzymatically dechorionated with Pronase and then fixed in 1% formaldehyde in embryo medium for 15 min at room temperature. For each immunoprecipitation experiment, approximately 200 fixed embryos were homogenized in cell lysis buffer and incubated for 15 min on ice. Nuclei were collected by centrifugation, resuspended in 200 µl of nuclei lysis buffer, incubated for 10 min on ice and then sonicated using Bioruptor (Cosmo Bio, Japan) to yield DNA fragments with an average size of 400–500 bases. The supernatant of the sonicated cells was diluted 10-fold with ChIP dilution buffer (50 mM Tris-HCl [pH 8.0], 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate). 950 µl of the diluted lysate was then incubated overnight at 4°C with Protein G Dynabeads (Invitrogen) that had been prebound to 2 µg of anti-SOX2 antibody (AF2018; R&D, MN, USA). The same volume of the lysate was precipitated with normal goat IgG as a negative control. 100 µl of the lysate was used as an input control. Beads were washed four times with RIPA buffer and once with TE buffer containing 50 mM NaCl. Bound complexes were eluted from the beads and cross-links were reversed in 200 µl of elution buffer for six hours at 65°C. Eluted DNA was then purified by treatment with RNase A, followed by proteinase K digestion, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Precipitated DNA was resuspended in 30 µl of TE buffer and 1 µl of the DNA suspension was used as a template for ChiP-PCR, which was performed using ExTaq polymerase (Takara) in 20 µl ExTaq buffer containing 0.17 mM crenosol red, 10% sucrose and the primers listed in Table S6. PCR products were separated in a 2% agarose gel (1.5% Methaphor agarose/0.5% agarose) and stained with SYBR green I.

Regulatory sequence analysis of the hesx1, cyp26a1, and pcdh18a genes

The fragments of the zebrafish hesx1 promoter (Zv8_NA6682:4150–4984, 835 bp), cyp26a1 promoter (Zv8_chr5:12933416–9335090, 1.7 kb) and pcdh18a conserved upstream sequence (Zv7_chr1:9017351–9017762, 412 bp) were amplified by PCR from the zebrafish genome using following primers (linker sequences that incorporate restriction sites are indicated by lowercase):

- **hesx1 promoter**
  - gggagactGGTCAAAACGCTCTCCAAACGTGAT and ggggtcgacCTCAAAGTCCTTTATTTACCTCAGGT
  - cyp26a1 promoter
  - gggagactAGTATTCCCCGTCGCCATG and ggggtcgacGTT
Figure S3 B1 sox activity is efficiently eliminated from the zebralfish embryo by B1 sox QKD. (A) Schematic representation of the NES30-TK200-nsVenus/lsec1 transgene construct used. NES30 is the 30-bp nestin enhancer core sequence, which is composed of SOX and POU binding sites. (B) NES30-driven nsVenus expression (controls in upper panels) was abolished by injection of the MOs for QKD (lower panels), confirming effective depletion of B1 SOX activity from the embryo. Found at: doi:10.1371/journal.pgen.1000936.s006 (1.83 MB TIF)

Figure S4 Effects of the B1 sox QKD revealed using transgenic lines with the GAL4-mediated reporter expression in the CNS and axial mesoderm. (A) Effects of QKD on CNS development were examined using the GAL4 enhancer trap line, which reports the activity of nr2f2 (confy) neural enhancers. Double transgenic embryos harboring GALAVPmad2 (#5m-3) and UAS:DrRedExDR normally show reporter expression in the CNS, which mimics neural nr2f2 expression. Injection of the MOs for QKD abolished this reporter expression, indicating impairment of CNS development. (B) Effects of QKD on axial mesoderm development, examined using the GALAVP16/hg/nc) line. Double transgenic embryos harboring GALAVP16/hg/nc) transgene and UAS:DrRedEx show reporter expression in the hatching gland (hg) and notochord (nc). Strong expression of UAS:DrRedEx was observed after 1 dpf in this line. Injection of the MOs for QKD did not reduce reporter expression, indicating normal axial mesoderm differentiation in the morphants. However, hatching gland cells remained as a single ball-like structure in the heads of the severe morphants (arrowhead). Found at: doi:10.1371/journal.pgen.1000936.s007 (1.11 MB TIF)

Figure S5 Gene expression profiles of the QKD embryos analyzed by microarray. Venn diagrams of genes that were found to be downregulated (A) and upregulated (B) in the B1 sox QKD embryos. Microarray analysis was carried out to compare gene expression profiles at the 30%E, 75%E, and tailbud stages between wild-type embryos and the QKD embryos. The numbers of Allofematrix zebralfish microarray probes that showed more than a twofold decrease (Table S2) or increase (Table S3) in the QKD embryos are shown. Annotated genes that were altered in all three stages are listed on the bottom in order of fold change at 75%E. Found at: doi:10.1371/journal.pgen.1000936.s008 (0.39 MB TIF)

Figure S6 Phenotypes of pdel18a and pdel18b knockdowns. Bright-field images of live embryos at 10–11.5 hpf and 31–31.5 hpf are shown. (A) Uninjected control (Ctrl) embryos. (B–D) Single and double knockdowns of pdel18a/18b. Moderate amounts of MO (3.6 ng) were used for single KD (B,C) and a 1:1 mixture of two MOs (3.6 ng each) was injected for double KD (D). Double knockdown embryos showed a shorter anteroposterior axis than the single knockdown embryos, indicative of more severe defects in C&E movements and suggesting a degree of functional redundancy between pdel18a and pdel18b. Found at: doi:10.1371/journal.pgen.1000936.s009 (0.42 MB TIF)

Figure S7 Expression of stmn2a and pou5f1 in the QKD embryos. (A) Expression of the stmn2a gene is upregulated in the QKD embryos at the shield stage. (B) Expression levels of pou5f1 were not affected in the B1 sox QKD embryos (our microarray data), whereas its expression domain was ventrally expanded at 75%E. Found at: doi:10.1371/journal.pgen.1000936.s010 (1.11 MB TIF)

Table S1 Summary of gene expression analysis using in situ hybridization and/or RT-PCR. Found at: doi:10.1371/journal.pgen.1000936.s0003 (0.67 MB TIF)
Table S2  Downregulated genes at 30% epiboly, 75% epiboly, and tailbud stages. Found at: doi:10.1371/journal.pgen.1000936.s009 (0.13 MB XLS)

Table S3  Upregulated genes at 30% epiboly, 75% epiboly, and tailbud stages. Found at: doi:10.1371/journal.pgen.1000936.s010 (0.12 MB XLS)

Table S4  Morpholino antisense oligonucleotides, Found at: doi:10.1371/journal.pgen.1000936.s011 (0.11 MB DOC)

Table S5  Primers and conditions for RT-PCR. Found at: doi:10.1371/journal.pgen.1000936.s012 (0.08 MB DOC)

Table S6  Primers and conditions for ChIP-PCR. Found at: doi:10.1371/journal.pgen.1000936.s013 (0.04 MB DOC)

Table S7  Comparison of B1 SOX and Pou5f1 regulated genes. Found at: doi:10.1371/journal.pgen.1000936.s014 (0.07 MB XLS)

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Author Contributions
Conceived and designed the experiments: YO YK. Performed the experiments: YO EO YK. Analyzed the data: YO YK. Wrote the paper: YO HK YK.

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