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Original Article

XSu(H)2 is an essential factor for gene expression and morphogenesis of the *Xenopus* gastrula embryo

MOTOAKI ITO, TOMOHISA KATADA, SEIJI MIYATANI and TSUTOMU KINOSHITA*

Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Hyogo, Japan

ABSTRACT The CSL (CBF-1, Suppressor of Hairless, Lag-1) transcriptional factor is an important mediator of Notch signal transduction. It plays a key role in cell fate determination by cell-cell interaction. CSL functions as a transcriptional repressor before the activation of Notch signaling. However, once Notch signaling is activated, CSL is converted into a transcriptional activator. It remains unclear if CSL has any function during early development before neurogenesis, while transcriptional products exist from the maternal stage. Here, we analyzed the function of *Xenopus* Suppressor of Hairless (XSu(H)) using morpholino antisense oligonucleotides (MO), which interfere with the translation of transcripts. In *Xenopus* embryos, maternal transcripts of both *XSu(H)1* and *XSu(H)2* were ubiquitously observed until the blastula stage and thereafter only *XSu(H)1* was zygotically transcribed. Knockdown experiments with MO demonstrated that XSu(H)2 depletion caused a decrease in the expression of the *Xbrachyury, MyoD* and *JNK1* genes. Morphological and histological examinations indicated that XSu(H)2 depletion caused abnormal gastrulation, which resulted in severe defects of the notochord and somitic mesoderm. The effect of XSu(H)2-MO was completely rescued by co-injection of *XSu(H)2* mRNAs, but not by *XSu(H)1* mRNAs. *XESR-1*, a Notch signaling target gene, inhibited *Xbrachyury* expression. However, expression of the *XESR-1* gene was not induced by depletion of XSu(H)2. Co-injection of the dominant-negative form of *XESR-1* could not rescue the suppression of *Xbrachyury* expression in the XSu(H)2-depleted embryo. These results suggest that XSu(H)2 is involved in mesoderm formation and the cell movement of gastrula embryos in a different manner from the XESR-1-mediated Notch signaling pathway.

KEY WORDS: *Suppressor of Hairless, notch signaling, gastrulation, Xbrachyury, XESR-1*

Introduction

CSL (CBF-1, Suppressor of Hairless, Lag-1) is a highly conserved transcriptional factor from human to *C. elegans* (Schweisguth *et al.*, 1992; Amakawa *et al.*, 1993; Tun *et al.*, 1994; Christensen *et al.*, 1996; Wettstein"*et al.*, 1997). CSL functions as a transcriptional factor of Notch signaling transduction, which is essential for a variety of developmental processes, including asymmetric cell-fate decision and boundary formation (Artavanis-Tsakonas *et al.*, 1995). When Notch signaling is activated by binding ligands, the Delta and Serrate/Jagged family, the transmembrane domain of Notch receptor is proteolytically cleaved and the Notch intracellular domain (NICD) is released from the membrane. NICD then translocates into the nucleus and transactivates *Hairy/Enhancer of Split* (*E(spl)*) by association with CSL. In the absence of NICD, CSL act as transcriptional repressors by associating with Hairless, CtBP, Groucho, Histone deacetylase (HDAC) in *Drosophila* (Furiols and Bray, 2000; Morel *et al.*, 2001; Barolo *et al.*, 2000; 2002), or with CIR, SMART, SKIP, HDAC1 in mammalian tissues (Chen and Evans, 1995; Horlein *et al.*, 1995; Kao *et al.*, 1998; Hisheh *et al.*, 1999; Zhou and Hayward, 2001); however, binding of NICD causes CSL to convert into transcriptional activators (Artavanis-Tsakonas *et al.*, 1995; Lai, 2002).

Null mutant mice of RBP-Jk, a mouse homologue of CSL, show severe growth retardation at 8.5 days of gestation and defective neurogenesis and somitogenesis at 9.5 days of gestation and then become lethal before day 10.5 of embryogenesis (Oka *et al.*, 1995). This lethal phase of RBP-Jk null mutant mice appears at an earlier stage than in Notch1 null mutant mice

Abbreviations used in this paper: CSL, CBF-1, Suppressor of Hairless, Lag-1 transcription factor; MO, morpholino antisense oligonucleotide; XSu(H), *Xenopus* Suppressor of Hairless.

***Address correspondence to:** Dr. Tsutomu Kinoshita. Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Gakuen 2-1, Sanda, Hyogo 669-1337, Japan. Fax: +81-79-565-9077. e-mail: tom@ksc.kwansei.ac.jp

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(Conlon *et al*., 1995), suggesting that CSL has a different function from Notch-dependent signaling. The Notch-independent CSL function has been indicated in the *Drosophila* mechanoreceptor (Barolo *et al*., 2000) who reported that Su(H) maintains its own activity by auto-activating the socket cell-specific transcriptional enhancer on the Su(H) gene and this auto-activation does not require continued Notch signaling. Notch signaling is involved in various cell fate decisions such as neurogenesis, somitogenesis and T cell / B cell differentiation (Furukawa *et al*., 1992; Han *et al*., 2002; Yamamoto *et al*., 2003). Expressions of chick Notch1 and Delta are detected at epiblast in early gastrula (Capriol *et al*., 2002) and X-Delta-1 (Kuroda *et al*., 1999; Wittenberger *et al*., 1999), dndeltaC, dndeltaD (Haddon *et al*., 1998; Smithers *et al*., 2000) and dnnotch1 (Bierkamp and Campos-Ortega, 1993) are detected at the marginal zone, which are premesodermal cells in *Xenopus* and zebrafish embryos. *Xotch* is expressed ubiquitously at the early gastrula stage and is required for muscle formation (Coffman'*et al*., 1993). Notch signaling is involved in notochord patterning in the midline and L-R patterning in the lateral plate mesoderm (Latimer *et al*., 2002; Lopez *et al*., 2003, 2005; Raya *et al*., 2004); however, there is little information about the function of Notch signaling in germ layer formation during earliest embryogenesis. These results suggest that Notch signaling plays an active role in the cell fate decision in gastrula embryos. In this study, we examined the role of CSL in germ layer formation during *Xenopus* early embryogenesis. In *Xenopus*, two CSL homologues, *XSu(H)1* and *XSu(H)2* have been cloned. XSu(H)1 has been reported as a down-stream factor of Notch signaling but XSu(H)2 has not been analyzed yet (Wettstein *et al*., 1997). Notch/XSu(H)1 signaling-induced XESR-1, a Notch signaling target gene, inhibits primary neurogenesis (Wettstein *et al*., 1997; Kiyota and Kinoshita, 2002). Here we report that XSu(H)2 can regulate the expression of *XESR-1* and that depletion of XSu(H)2 causes the down-regulation of zygotically expressed genes in mesoderm formation, which results in abnormal gastrulation. We also show that XSu(H)2 regulates *Xbrachyury* expression through a pathway different from Notch signaling via XESR-1.

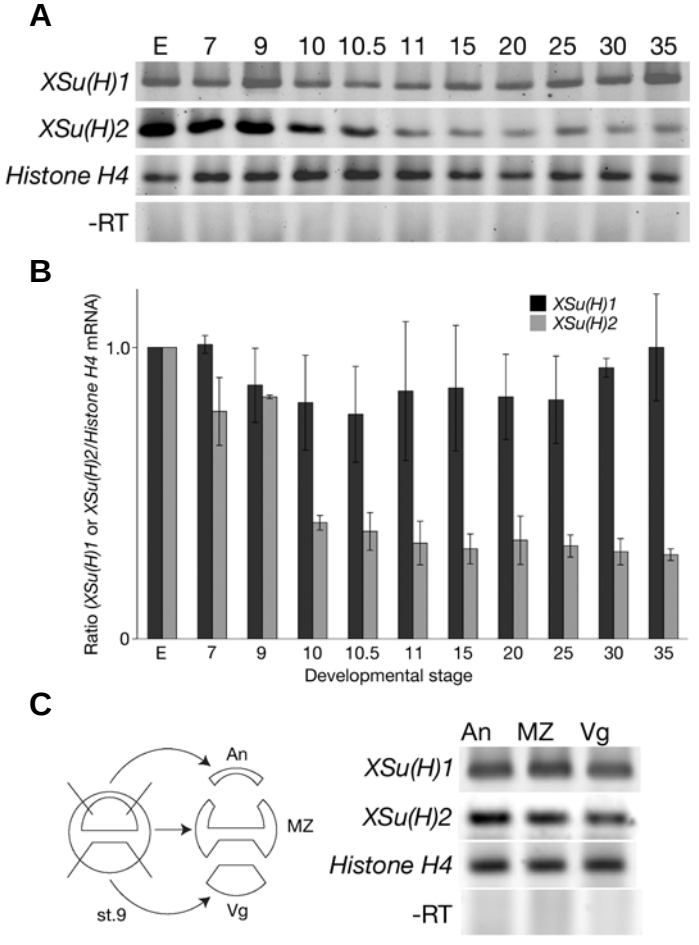

Fig. 1. The temporal expression pattern of *XSu(H)1* and *XSu(H)2*. (A) *Developmental profile of* XSu(H)1 *and* XSu(H)2 *expression. Both* XSu(H)1 *and* XSu(H)2 *transcripts were detected ubiquitously from unfertilized eggs (E) to stage 35. Enriched maternal transcripts of* XSu(H)2 *were recognized from unfertilized eggs to the gastrula stage.* Histone H4 *was used as a loading control. -RT, PCR without reverse transcriptase.* **(B)** *Quantification of* XSu(H)1 *and* XSu(H)2 *expression at each stage. The vertical line indicates the relative value of* XSu(H)2/Histone H4 *ratio calculated with sample E as 1. Experiments were carried out in triplicate.* **(C)** *Distribution of* XSu(H)1 *and* XSu(H)2 *transcripts. Blastula stage (stage 9) embryos were dissected into animal cap (An), marginal zone (MZ) and vegetal cap (Vg) and gene expression was detected by RT-PCR.*

Results

The expression of XSu(H)2 *is different from that of* XSu(H)1

In *Xenopus*, *XSu(H)1* and *XSu(H)2* have been reported as CSL homologues. XSu(H)1 has an additional 20 amino acid residues at the N-terminal region of XSu(H)2, but otherwise they have an identical amino-acid sequence. First, we performed semi-quantitative RT-PCR in order to know the expression pattern of *XSu(H)1* and *XSu(H)2* during the early development of *Xenopus*. Transcriptional products of *XSu(H)1* were detected ubiquitously from the unfertilized egg to the tailbud stage embryo as in a previous report (Fig. 1A, B, Wettstein *et al*., 1997). Transcripts of *XSu(H)2* were also detected ubiquitously during all embryonic stages; however, enriched transcripts of *XSu(H)2* were detected maternally until the gastrula stage and thereafter sharply decreased to the same level as *XSu(H)1* (Fig. 1A, B). The ubiquitous distribution of *XSu(H)1* transcript has been reported (Wettstein *et al*., 1997), but that of XSu(H)2 is still unknown. In order to elucidate the distribution of *XSu(H)* transcripts, blastula embryos were dissected into three parts at stage 9 and semi-quantitative RT-PCR was performed for each extract. As shown in Figure 1C, both *XSu(H)1* and *XSu(H)2* were localized ubiquitously in the blastula embryo. These results suggest that XSu(H) plays a role during the early embryonic stages.

Translational inhibition of XSu(H)2 causes abnormal gastrulation and neural fold disorganization

We investigated whether XSu(H) is involved in early embryogenesis by using morpholino antisense oligonucleotides (MO). First, we tested whether the MOs prepared against XSu(H)1 and XSu(H)2 specifically inhibit the translation of each transcript (Fig. 2A). The expressions of myc-XSu(H)1 and myc-XSu(H)2 proteins were detected with anti-myc antibody (lanes 2, 6). XSu(H)1-MO and XSu(H)2-MO inhibited the translation of its own target (lanes 3, 7), but XSu(H)1-MO and XSu(H)2-MO did not inhibit the

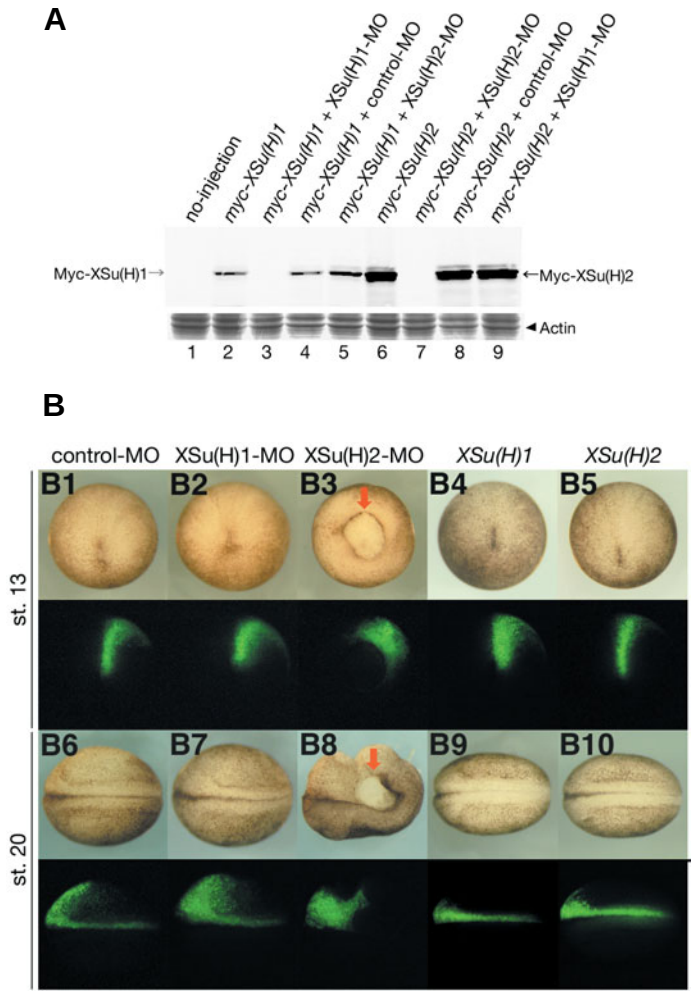

Fig. 2. Effect of XSu(H) morpholinos on early embryonic development. (A) *Specificity of morpholino oligonucleotides (MO). Five ng of* myc-XSu(H)1 *or* myc-XSu(H)2 *mRNA was injected into the animal pole of each blastomere of 2-cell stage with or without 25 ng of XSu(H)1-MO or XSu(H)2-MO. XSu(H)1 or XSu(H)2 protein was detected by anti-c-myc antibody at stage 10.5. 43 kDa actin bands were used as loading controls (Coomassie stained).* **(B)** *Phenotype of the MO-injected embryo. Embryos were injected with control-MO (B1, B6), XSu(H)1-MO (B2, B7), XSu(H)2-MO (B3, B8), mRNAs of* XSu(H)1 *(B4, B9) or* XSu(H)2 *(B5, B10) into one dorsal blastomere at the 4-cell stage and morphological changes were analyzed at stage 13 (B1-5) or stage 20 (B6-10). To identify the injection side of the embryo, 1 ng of* GFP *mRNA as the tracer was co-injected with each mRNA or MO. Upper panels (B1-5) show the vegetal view and lower panels (B6-10) show the dorsal view. XSu(H)2-depleted embryos showed delayed gastrulation (arrow in B3) and defective neurogenesis (arrow in B8), while XSu(H)1-depleted embryos developed normally (B2, B7).*

translation of *XSu(H)2* and *XSu(H)1*, respectively (lanes 5, 9). The results indicate that these MOs are able to specifically inhibit the translation of each XSu(H). Next, we observed the effect of MO on the early development of embryos. Morpholino oligonucleotide was injected into one blastomere of a four-cell-stage embryo and the injection side of MO was detected by co-injecting with *GFP* mRNA. Embryos injected with either 25 ng of XSu(H)1-MO or XSu(H)2-MO developed normally until the early gastrula stage (stage 10.5). Thereafter, however, the XSu(H)2-MO-injected embryos showed abnormal gastrulation and neural fold disorganization (Fig. 2B3, B8). These effects were not observed in embryos injected with XSu(H)1-MO nor control-MO (Fig. 2B1, B2, B6, B7; Table 1). In contrast, overexpression of *XSu(H)2* did not cause any developmental abnormality, which was the same as the overexpression of *XSu(H)1* (Fig. 2B4, B5, B9, B10) or *XSu(H)1* + *XSu(H)2* (data not shown). These results suggest that XSu(H)2 plays an important role from gastrulation to neurogenesis.

XSu(H)2 is essential for gastrulation and Xbrachyury *expression*

In order to examine the tissue affected by XSu(H)2-MO, histological analysis was performed on XSu(H)2-depleted embryos. Since severe defects of XSu(H)2 cause developmental arrest at the gastrula stage, low-dose XSu(H)2-MO (10 ng per embryo) was used in the histological examination. In stage 35 embryos injected with XSu(H)2-MO in one blastomere at the 2-cell stage, tissue defects were observed in the somite and notochord, but not in the neural tube on the injection side (100%, n=10) (Fig. 3A, B). These tissue defects were not detected either in the control-MO-injected embryo (100%, n=9) or in the XSu(H)1-MO-injected embryo (100%, n=10). Since morphological abnormalities in the XSu(H)2-depleted embryos first appeared during gastrulation, we analyzed the expression of genes essential for development in the early gastrula stage. As shown in Figure 3C, depletion of XSu(H)2 caused a remarkable decrease in *Xbrachyury*, *MyoD*, *Xvent1*, *chordin* and *JNK1* expression, which was not observed in the XSu(H)1-depleted embryo. Even under these conditions, *goosecoid* showed a normal expression both in XSu(H)1-MO- and XSu(H)2-MO-injected embryos. Overexpression of *XSu(H)1* or *XSu(H)2* had no effect on the expression of marker genes (Fig. 3C). These results indicate that XSu(H)2 is an essential factor in gastrulation, mesoderm formation and cell movement of gastrula embryos, which is quite different from the role of XSu(H)1.

Effect of XSu(H)2-MO is caused by the specific inhibition of XSu(H)2 function

In order to confirm whether XSu(H)2-MO specifically inhibits the translation of XSu(H)2 but not XSu(H)1, we performed rescue experiments by co-injection of *XSu(H)1* or *XSu(H)2* together with

TABLE 1

EFFECT OF XSU(H)-MO ON GASTRULATION AND TYPE-SPECIFIC RESCUE

Injected sample (ng)	Total number of embryos	Phenotype (%) Gastrulation defect	Unaffected
control-MO (25)	63	0	100
XSu(H)1-MO (25)	52	0	100
XSu(H)2-MO (25)	56	100	0
XSu(H)2-MO (25) + *Δ5′UTR XSu(H)1* (2.0)	25	100	0
XSu(H)2-MO (25) + *Δ5′UTR XSu(H)1* (5.0)	25	100	0
XSu(H)2-MO (25) + *Δ5′UTR XSu(H)2* (2.0)	32	44	56
XSu(H)2-MO (25) + *Δ5′UTR XSu(H)2* (5.0)	40	10	90

Twenty-five ng of morpholino antisense oligonucleotides (MO) were injected into one dorsal blastomere of 4-cell stage embryo, with or without 5′UTR-deleted mRNA of *XSu(H)1* or *XSu(H)2*. The injected embryos were cultured at 18°C until stage 11, when gastrulation defect was examined on each embryo.

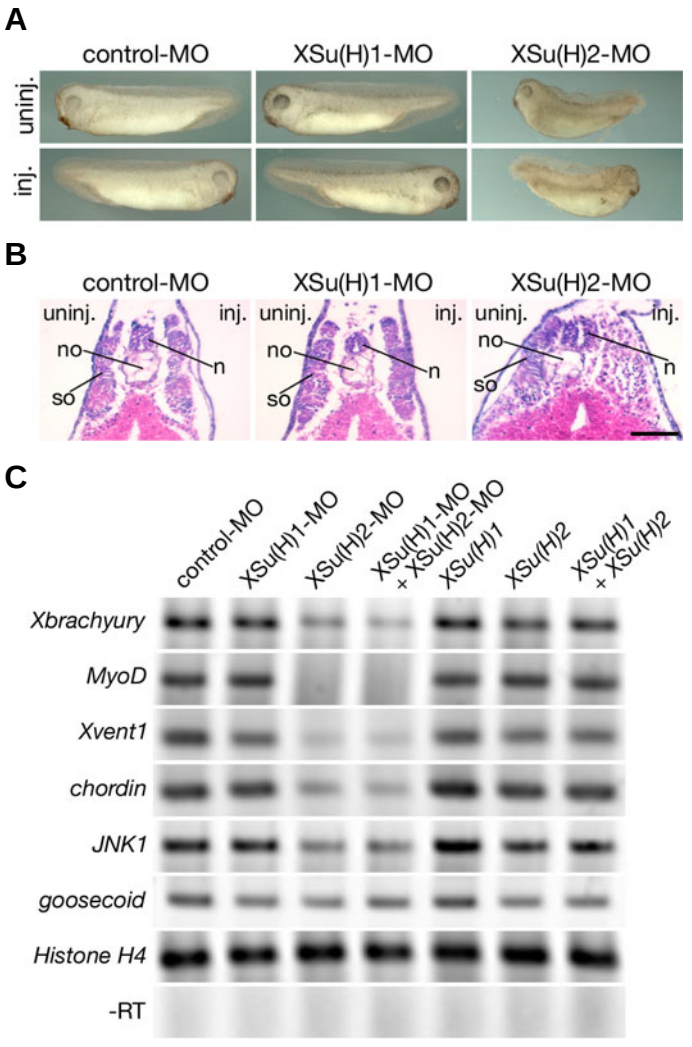

Fig. 3. Histological examination of XSu(H)2-depleted embryos. ***(A)*** *Morphological change of XSu(H)2-depleted embryos. Dwarf embryos were induced by XSu(H)2-MO but not by XSu(H)1-MO.* ***(B)*** *Cross section of XSu(H)2-depleted embryos. Histological analysis shows that embryos injected with XSu(H)2-MO had tissue defects in the somite (so) and notochord (no) without lacking a neural tube (n). Ten ng of each MO was injected into the marginal zone of one blastomere at the 2-cell stage. The injected embryos were fixed at stage 35-36 for histological examination. Scale bar indicates 100 μm.* ***(C)*** *Gene expression in XSu(H)2-depleted embryos. Twenty five ng of XSu(H)1-MO or XSu(H)2-MO was injected into the marginal zone of both blastomeres at the 2-cell stage. The injected embryos were sacrificed at stage 10.5 for quantitative RT-PCR. XSu(H)2-MO reduced* Xbrachyury, MyoD, Xvent1, chordin and JNK1 *expressions, but had no effect on* goosecoid *expression in gastrula stage embryos. XSu(H)1-MO,* XSu(H)1 XSu(H)2, XSu(H)1 + XSu(H)2 *showed no effect on the gene expression of mesodermal markers.*

XSu(H)2-MO. Since XSu(H)2-MO recognizes 5'UTR sequences just upstream of the first methionine, 5'UTR-deleted mRNA of *XSu(H)* (*Δ5'UTR-XSu(H)*) was used as a rescue molecule. Abnormal development caused by XSu(H)2-MO injection was completely rescued by co-injection of *Δ5'UTR-XSu(H)2* (Fig. 4A1, A2, A4), but not by *Δ5'UTR-XSu(H)1* (Fig. 4A3). Specific rescue by co-injection of *Δ5'UTR-XSu(H)2* was also confirmed by the recovery of *Xbrachyury* expression only in the embryo injected with XSu(H)2-MO and *Δ5'UTR-XSu(H)2* (Fig. 4B). This recovery occurred in a dose-dependent manner (Table 1). These results suggest that abnormal gastrulation is caused specifically by the depletion of XSu(H)2.

XSu(H)2-MO-induced suppression of Xbrachyury *can not be rescued by Notch signaling*

It is known that XSu(H)1 functions as a transcriptional factor under Notch signaling during neurogenesis (Wettstein *et al*., 1997), while it is unknown whether XSu(H)2 functions as a component of Notch signaling. To test the function of XSu(H)2, we made two constructs: XSu(H)2ANK, which is an active form of XSu(H)2 fused with the ankyrin region of Notch intracellular domain (NICD) and XSu(H)2DBM, which is a DNA-binding mutant prepared as a competitive inhibitor of Notch signaling by trapping NICD. Using these constructs, we examined the effect of XSu(H)2ANK and XSu(H)2DBM on the expression of *XESR-1*, a Notch signaling target gene. As shown in Figure 5A, *XSu(H)2ANK* activated the expression of *XESR-1*, whereas *XSu(H)2DBM* inhibited it as well as XSu(H)2-MO. These results are consistent with the effects of *XESR-1* by XSu(H)1 (Wettestein *et al*., 1997). At the same time, these results indicate that XSu(H)2 functions as a transcriptional factor under Notch signaling. Next, we examined whether Notch signaling is activated via endogenous XSu(H)1 even in the XSu(H)2-MO-injected embryo, because XSu(H)1 exists under depleted conditions of XSu(H)2. As shown in Figure 5A, *NICD*, the activated form of Notch signaling (Artavanis-Tsakonas *et al*., 1995; Wettstein *et al*., 1997), could activate the expression of *XESR-1* with the depletion of XSu(H)2, suggesting that Notch signaling can be activated under XSu(H)2-depleted conditions.

If XSu(H)2-mediated Notch signaling has a role in mesoderm formation, there is a possibility that activation of Notch signaling can rescue the decrease of *Xbrachyury* expression caused by XSu(H)2-MO injection. To test this possibility, we examined whether *NICD* injection can rescue the decrease of *Xbrachyury* expression in the XSu(H)2-MO-injected gastrula embryo. In contrast with the complete rescue by co-injection of *Δ5'UTR XSu(H)2* (Fig. 5B1-B3), *NICD* could not rescue the XSu(H)2-MO-induced suppression of *Xbrachyury* expression (Fig. 5B5; Table 2). The effect of XSu(H)2-MO on *Xbrachyury* was examined in an animal

TABLE 2

EFFECT OF *NICD* ON THE DECREASE OF *XBRACHYURY* EXPRESSION CAUSED BY XSU(H)2-MO

Injected sample (ng)	Total number of embryos	Xbrachyury expression (%)	
		Decrease	Unaffected
control-MO (25)	36	0	100
XSu(H)2-MO (12.5)	35	28	72
XSu(H)2-MO (25)	29	100	0
XSu(H)2-MO (25) + *Δ5'UTR XSu(H)2* (2.0)	32	16	84
XSu(H)2-MO (25) + *NICD* (2.0)	35	91	9
XSu(H)2 (2.0)	32	6	94
XSu(H)2DBM (2.0)	24	0	100
NICD (2.0)	31	0	100

XSu(H)2-MO was injected with mRNAs of *XSu(H)2* or *NICD* into one dorsal blastomere of 2-cell stage embryo. The injected embryos cultured at 18 °C were fixed with 1X MEMFA at stage 10.5 and gene expression of *Xbrachyury* was examined by whole-mount *in situ* hybridization.

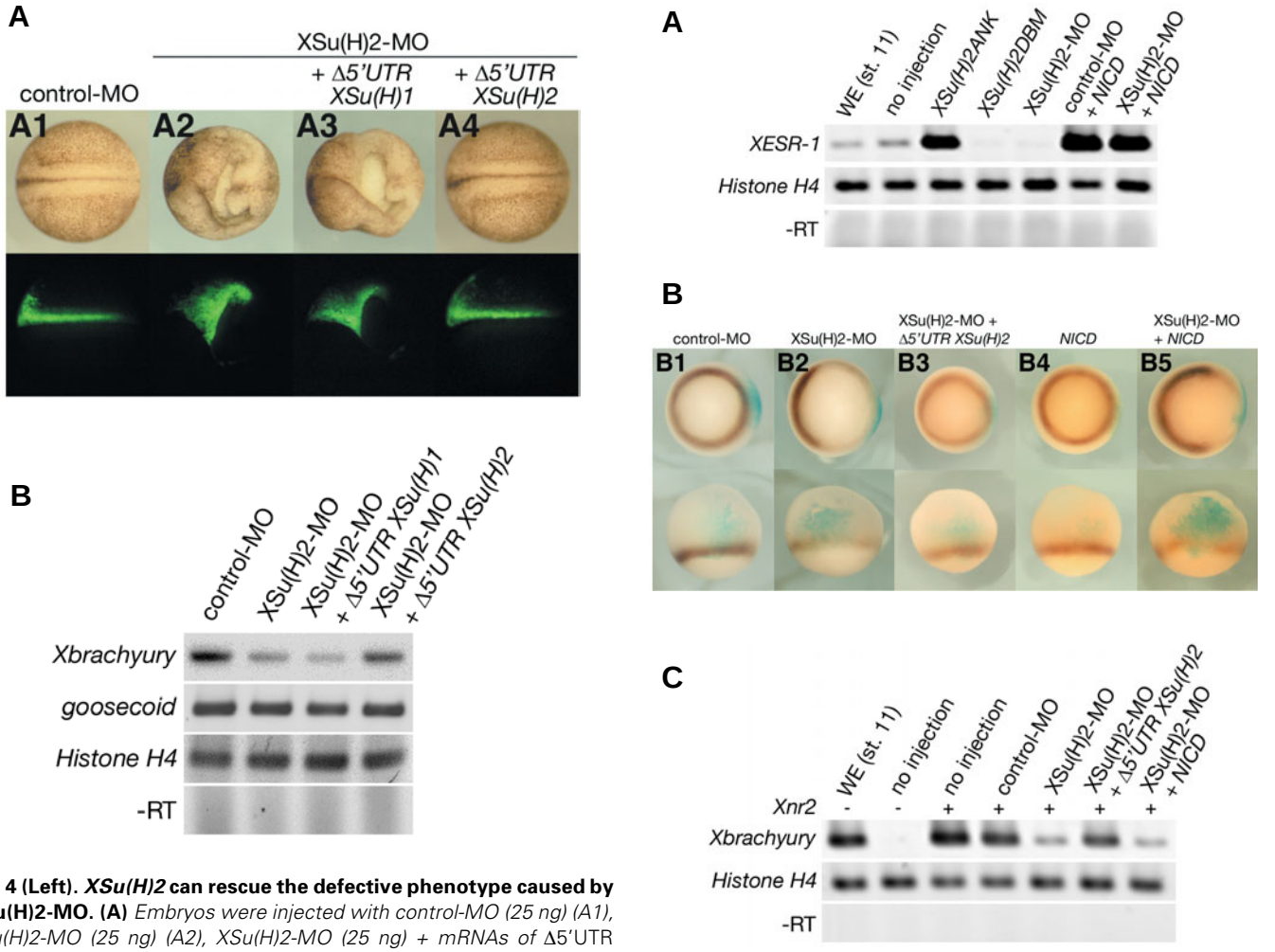

Fig. 4 (Left). *XSu(H)2* can rescue the defective phenotype caused by XSu(H)2-MO. (A) *Embryos were injected with control-MO (25 ng) (A1), XSu(H)2-MO (25 ng) (A2), XSu(H)2-MO (25 ng) + mRNAs of* Δ5'UTR XSu(H)1 *(2 ng or 5 ng) (A3) or XSu(H)2-MO (25 ng) + mRNAs of* Δ5'UTR XSu(H)2 *(2 ng or 5 ng) (A4) into one dorsal blastomere at the 4-cell stage and morphological phenotype was examined at stage 20. Upper panel shows the dorsal view and lower panel indicates the injected side shown by GFP fluorescence on the same view.* **(B)** *Embryos were injected into the marginal zone of both blastomeres at the 2-cell stage and used for the assay of quantitative RT-PCR at stage 11. The defective neurogenesis and the reduction of* Xbrachyury *expression caused by XSu(H)2-MO were rescued by* Δ5'UTR XSu(H)2 *(A4, B), but not by* Δ5'UTR XSu(H)1 *(A3, B).*

Fig. 5 (Right). *Xbrachyury* expression suppressed by XSu(H)2-MO cannot be rescued by activation of Notch signaling. (A) *Embryos were injected with* XSu(H)2ANK *(2 ng),* XSu(H)2DBM *(2 ng), XSu(H)2-MO (50 ng), control-MO (50 ng) +* NICD *(2 ng) or XSu(H)2-MO (50 ng) +* NICD *(2 ng). Animal cap explants were isolated from the injected embryos at stage 8 and were cultured until stage 11 for RT-PCR.* XSu(H)2ANK *as an activation construct of Notch signaling increased the gene expression of* XESR-1, *while* XSu(H)2DBM *as a dominant-negative form of Notch signaling inhibited it. Even under depleted XSu(H)2 protein,* NICD *could activate the expression of* XESR-1. **(B)** *Embryos were injected with control-MO (B1), XSu(H)2-MO (B2), XSu(H)2-MO + mRNAs of* Δ5'UTR XSu(H)2 *(B3), mRNAs of* NICD *(B4) or XSu(H)2-MO + mRNAs of* NICD *(B5) into the marginal zone of one blastomere at the 2-cell stage. The injected embryos were cultured until stage 10.5 and the expression of* Xbrachyury *was examined using whole-mount* in situ *hybridization. All embryos were injected with 1 ng of* β-galactosidase *mRNA as a tracer of the injection side. The injected side was colored blue by staining the activity of β-galactosidase. The expression of* Xbrachyury *was colored brown. Upper and lower panels show vegetal and lateral views, respectively. Suppression of* Xbrachyury *gene expression by XSu(H)2-MO occurred widely (B2) and could be rescued by co-injection of* Δ5'UTR XSu(H)2 *(B3); however, activation of Notch signaling by* NICD *could not rescue the XSu(H)2-MO-induced reduction of* Xbrachyury *expression (B5).* NICD *alone did not suppress* Xbrachyury *expression (B4).* **(C)** *Synthesized RNAs of 50 pg* Xnr2 *were injected into the animal pole of 2-cell stage embryos with 50 ng various MO or 2 ng mRNAs. Animal caps were dissected from the injected embryos at stage 8 and were harvested at stage 11 for RT-PCR analysis. XSu(H)2-MO reduced* Xbrachyury *expression induced by* Xnr2. Δ5'UTR XSu(H)2, *but not* NICD *could rescue the XSu(H)2-MO-induced suppression of* Xbrachyury *gene expression.*

cap assay, where *Xbrachyury* expression induced by *Xnr2* was suppressed by the injection of XSu(H)2-MO (Fig. 5C). The suppression of *Xbrachyury* expression by XSu(H)2-MO was rescued by co-injection with *Δ5'UTR XSu(H)2* but not with *NICD* (Fig. 5C). The activation of Notch signaling by *NICD* injection alone did not affect the expression of *Xbrachyury* (Fig. 5B4). *NICD* did not induce the expression of *Xbrachyury* in animal cap without a mesoderm-inducing factor (data not shown). These results show

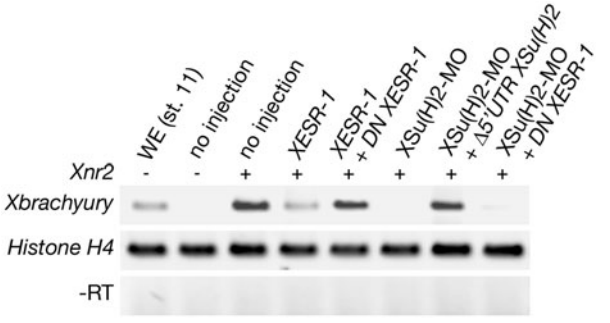

Fig. 6. *Xbrachyury* expression by XSu(H)2 is independent of regulation by XESR-1. *Synthesized RNAs of 50 pg* Xnr2 *were injected into the animal pole of 2-cell stage embryos together with* XESR-1 *(1 ng)),* XESR-1 *(1 ng) +* DN XESR-1 *(4 ng), XSu(H)2-MO (50 ng), XSu(H)2-MO (50 ng) +* Δ5′UTR XSu(H)2 *(4 ng), XSu(H)2-MO (50 ng) +* DN XESR-1 *(4 ng). Animal caps were dissected from the injected embryos at stage 8 and were cultured until stage 11 and then the gene expression of* Xbrachyury *was examined using quantitative RT-PCR. Overexpression of* XESR-1 *reduced* Xbrachyury *induced by* Xnr2*. This inhibition could be rescued by co-injection with* DN XESR-1*. The reduction of* Xbrachyury *expression caused by XSu(H)2-MO could be rescued by co-injection of* Δ5′UTR XSu(H)2*, but not dominant-negative* XESR-1*,* DN XESR-1*.*

that the downregulation of *Xbrachyury* expression caused by XSu(H)2-MO is not recovered by activating Notch signaling.

XSu(H)2 regulates Xbrachyury *expression without transactivation of* XESR-1

From the results of Figure 5, XSu(H)2 seems to regulate the expression of *Xbrachyury* in a different manner from Notch signaling. To test whether XSu(H)2 regulates the expression of *Xbrachyury* through XESR-1, we investigated the effect of XESR-1 on *Xbrachyury* expression in the animal cap assay. As shown in Figure 6, overexpression of *XESR-1* intensely suppressed *Xnr2*-induced *Xbrachyury* expression, showing that XESR-1, a target gene of Notch signaling, is a negative regulator of *Xbrachyury* expression. Next, we examined whether XSu(H)2 depletion activates *XESR-1*, which results in the suppression of *Xbrachyury* expression. In order to test this idea, we constructed *DN XESR-1*, the dominant-negative form of *XESR-1* lacking the C-terminal WRPW motif (Fisher *et al*., 1996; Giebel and Campos-Ortega, 1997). Prior to using *DN XESR-1*, we checked the effect of this molecule on primary neurogenesis. The injection of this construct into embryos caused an increased expression of *N-tubulin* (data not shown). Then, we examined whether *DN XESR-1* injection can rescue the decrease of *Xbrachyury* expression in the XSu(H)2-MO-injected animal cap. As shown in Figure 6, *DN XESR-1* could not rescue the downregulation of *Xbrachyury* caused by XSu(H)2-MO, while it could rescue that caused by *XESR-1*. These results show that XSu(H)2-MO-induced suppression of *Xbrachyury* expression is not caused by the activation of *XESR-1.*

Discussion

XSu(H)1 is known to function as a transcriptional factor of Notch signaling that controls the neurogenic pathway. Here, we demonstrated that not XSu(H)1 but XSu(H)2 plays an important role in the gastrulation of *Xenopus* embryos.

XSu(H)2 has a different function from XSu(H)1 during early embryogenesis

XSu(H)2 knockdown embryos showed remarkably abnormal phenotypes, whereas the depletion of XSu(H)1 caused no change in normal development at the gastrula stage (Fig. 2B2, B3, B7, B8). The decrease of *Xbrachyury* expression caused by XSu(H)2-MO could be rescued by *Δ5′UTR XSu(H)2*, but not by *Δ5′UTR XSu(H)1* (Fig. 4A3, A4, B). Regardless of the same expression pattern of both genes (Fig. 1B), these results revealed that XSu(H)2 may have a different function from XSu(H)1. XSu(H)1 has 20 additional amino acids at its N-terminus, different from XSu(H)2. Moreover, XSu(H)1 has different 5'UTRs from XSu(H)2. Xpygopus-alpha and -beta are transcriptional factors and their structures are similar to XSu(H)1 and XSu(H)2 (Lake and Kao, 2003). As compared with Xpygopus-beta, Xpygopus-alpha has 21 additional N-terminal residues and shows a different temporal expression pattern (Lake and Kao, 2003). In *Xenopus*, it is likely that these isoforms play different roles during early development, since the additional 20 amino-acid region is able to interact with some factors.

Deficiency of XSu(H)2 protein by XSu(H)2-MO caused a decreased expression of mesodermal markers, which resulted in abnormal gastrulation (Fig. 2B3, B8, 3C). JNK1 is involved in convergent extension of the Wnt/JNK pathway in a mesoderm-independent manner (Yamanaka *et al*., 2002). It is reported that Xbrachyury functions as a switch between cell migration and convergent extension (Kwan and Kirschner, 2003). The decrease of *JNK1* expression caused by XSu(H)2-MO (Fig. 3C) may result from the reduction of *Xbrachyury* expression, because it has been reported that *Xbrachyury* is required for convergent extension movements and functions upstream of Wnt11, a ligand of the Wnt/JNK pathway (Smith, 2000; Tada and Smith, 2000; Kuhl, 2002; Kwan and Kirschner, 2003; Carron *et al*., 2005). Thus, abnormal development in the XSu(H)2-depleted embryo may result from defective mesoderm formation and cell movement caused by the lack of *Xbrachyury* expression; however, it is also possible that XSu(H)2 regulates both *Xbrachyury* and *JNK* expression independently. Further examination is necessary to clarify this possibility. Gastrulation defects are not caused by the inhibition of Notch signaling using a dominant-negative Notch ligand, X-Delta-1^{stu} (data not shown) and a dominant-negative Notch component, C-terminus-deleted Mastermind (Katada and Kinoshita, 2003). These results suggest that abnormal gastrulation caused by the depletion of XSu(H)2 occurs in a Notch signaling-independent manner.

The injection of XSu(H)1-MO had no effect on gastrulation, but induced the up-regulation of the primary neuronal marker, *N-tubulin* (data not shown). This is consistent with the inhibition of Notch signaling, which causes excessive primary neurogenesis (Greenwald, 1994; Wettstein *et al*., 1997; Kiyota and Kinoshita, 2002). These results suggest that XSu(H)1 functions as a down-stream factor of Notch signal transduction during primary neurogenesis, but not at the gastrula stage.

In the study of somitogenesis, overexpression of *XSu(H)1* leads to the defective segmentation of somites and alters the

segmented expression pattern of *XESR-5* and *X-Delta-2* (Gautier-Courteille *et al*., 2004). Inhibition of Notch signaling by expressing a dominant-negative mutant of XSu(H)1, *XSu(H)1DBM*, also caused segmentation defects and decreased the expression of *XESR-5* and *X-Dellta-2* (Jen *et al*., 1999). In this study, however, regardless of the clear knockdown effect induced by MO, overexpression of both *XSu(H)1* and *XSu(H)2* did not show a remarkable phenotype (Fig. 2B4, B5, B9, B10). Since CSL is thought to function as a mediator of transcriptional co-activators or transcriptional co-repressors (Lai, 2002), excessive co-factors other than XSu(H) are probably needed to induce the remarkable phenotype in the overexpression of XSu(H)1 and XSu(H)2.

Regulation of Xbrachyury *by XSu(H)2*

We showed that XSu(H)2 is involved in the regulation of *Xbrachyury* expression (Fig. 4A3, A4, B). In *Ciona*, CSL homologue Ci-Su(H) binds the CSL binding site on the *brachyury* promoter and directly activates the transcription of *brachyury* (Corbo *et al*., 1998). This is consistent with our result that *Xbrachyury* expression is repressed by the depletion of XSu(H)2. Notochordal cells in *Ciona* increase in number when X-Notch-1 is overexpressed in notochords using the *forkhead* promoter of *Ciona* (Corbo *et al*., 1998). In our experiment, however, overexpression of *NICD* could not increase the gene expression of *Xbrachyury* (Fig. 5B4), suggesting that XSu(H)2 is necessary but not enough to induce *Xbrachyury* expression.

In recent studies, the activation of Notch signaling by *NICD* decreases notochordal markers, such as *Xbrachyury* and *chordin* expression, in the dorsal midline during the *Xenopus* neurula stage (Lopez *et al*., 2003). Other studies indicate that Notch signaling is involved in the midline structure in zebrafish (Appel *et al*., 1999; Latimer *et al*., 2002). In this study, however, we examined the effect of XSu(H)2-MO on *Xbrachyury* expression not in the midline but in the entire marginal zone (presumptive mesodermal cells) at the early gastrula stage. XSu(H)2 at the gastrula stage may have a different function from Notch signaling at a later stage. Further study is required to understand the role of XSu(H)2 in *Xbrachyury* expression.

Function of XSu(H)2 in germ layer formation

RBP-Jk null mutant mice show abnormal somitogenesis and neurogenesis and die before day 10.5 of embryogenesis (Oka *et al*., 1995), indicating that CSL is an essential factor in early embryogenesis. RBP-Jk null mutant mice die much earlier than Notch1 null mutant mice (Conlon *et al*., 1995), suggesting that CSL plays an important role in early embryogenesis through a different pathway from Notch signaling. Recently, it was revealed that Notch signaling is involved in the cell fate determination of endomesoderm and mesoderm and is essential for mesoderm formation. In sea urchin embryos, it has been reported that activation of Notch signaling increased the non-skeletogenic mesoderm and the suppression of Notch signaling caused complete defect of the non-skeletogenic mesoderm (Sheerwood and McClay, 2001; Sweet *et al*., 2002). If this is the case in *Xenopus* embryos, abnormal gastrulation caused by XSu(H)2-MO may come from mesoderm defects induced by the suppression of Notch signaling. In *Xenopus* embryos, however, overexpression of *NICD* did not increase *Xbrachyury* expression (Fig. 5B4) and suppression of Notch signaling by *XSu(H)1/2DBM* could not affect the gene expression of *Xbrachyury* (data not shown). These results suggest that XSu(H)2 plays a role in *Xenopus* gastrulation in a Notch-independent manner.

In *Xenopus*, *X-Delta-1* and *XMyoD* are expressed in the marginal zone of early gastrula embryos. XMyoD stimulates *X-Delta-1* expression, whereas X-Delta-1 inhibits *XMyoD* expression (Kuroda *et al*., 1999; Wittenberger *et al*., 1999). Activation of Notch signaling causes an increase in the number of neural and muscle cells in *Xenopus* embryos (Coffman *et al*., 1993); however, it remains unclear how Notch signaling is involved in mesoderm formation in *Xenopus*. In zebrafish, it has been reported that the activation of Notch signaling decreases endodermal tissues, but the repression of Notch signaling cannot increase endodermal tissues (Kikuchi *et al*., 2004). In this study, we demonstrated the essential role of XSu(H)2 in *Xenopus* mesoderm formation, but could not show the molecular mechanism of XSu(H)2–dependent gastrulation.

XSu(H)2 regulates mesoderm formation without activation of XESR-1

Recent studies show that CSL interacts with NICD through a hydrophobic pocket on the beta-trefoil domain (BTD) of CSL (Nam *et al*., 2006; Wilson and Kovall, 2006). BTD is conserved between XSu(H)1 and XSu(H)2; therefore, it is thought that XSu(H)2-DBM can inhibit the Notch signaling pathway by trapping NICD, as shown in XSu(H)1DBM in a previous study (Wettstein *et al*., 1997). In fact, *XSu(H)2-DBM* could decrease *XESR-1* expression (Fig. 5A). In our study, either the activation of Notch signaling by *NICD* (Fig. 5B4) or the suppression of Notch signaling by *XSu(H)2DBM* (data not shown) showed no effect on *Xbrachyury* expression. Nevertheless, the XSu(H)2-knockdown embryo showed the downregulation of *Xbrachyury* expression and severe gastrulation defects. Judging from these results, it is likely that endogenous XSu(H)2 can regulate the transcription of *Xbrachyury* without NICD. Since endogenous XSu(H)2 exists under the *XSu(H)2DBM*-injected condition, *Xbrachyury* expression must be induced even in the *XSu(H)2DBM*-injected embryo. These results suggest that XSu(H)2 regulates *Xbrachyury* expression through a mechanism other than Notch signal transduction.

Overexpression of *XESR-1*, a target gene of Notch signaling, decreased *Xbrachyury* expression (Fig. 6), whereas *NICD* showed no effect (Fig. 5B4). We cannot explain why *NICD* showed no effect on the expression of *Xbrachyury*, because *NICD* could induce the gene expression of *XESR-1* (Fig. 5A). In contrast, XSu(H)2-MO suppressed *Xbrachyury* expression without the gene expression of *XESR-1* (Fig. 5A). *DN XESR-1*, a dominant-negative form of XESR-1, could rescue the decrease of *Xbrachyury* expression induced by *XESR-1*, but not by XSu(H)2-MO (Fig. 6). These results suggest that XSu(H)2 regulates *Xbrachyury* expression in a different manner from the XESR-1-mediated Notch signaling pathway. Further examination is necessary to clarify mechanism of XSu(H)2. Interestingly, in mouse embryos, NICD induces T-cell differentiation without the gene expression of *HES1* and *HES5*, mouse homologues of XESR-1, but its interference with B lymphocytes is partly mediated by HES genes (Kawamata *et al*., 2002). Both HES-dependent and HES-independent mechanisms must be

involved in the cell fate decision during animal development.

Materials and Methods

Eggs and embryos

Xenopus eggs were obtained by injecting human chorionic gonadoropin, gestron (Denka Seiyaku, Japan) into *Xenopus laevis* female and were fertilized with the testis isolated from *Xenopus laevis* male by surgical operation. Embryos were dejellied with 1% sodium thioglycollate and were cultured in 0.1X MMR (Marc's Modified Ringers, [10 mM NaCl; 0.2 mM $CaCl_2$; 0.5 mM HEPES, pH 7.5]). The developmental stages of embryos were determined by according to normal table of *Xenpus laevis* (Nieuwkoop and Faber, 1967). Animal cap injected samples was excised from the blastula stage (stage 8), cultured in 1x MMR including 100 mg/L kanamycin until stage 11 or 15.

Constructs and morpholino antisense oligo nucleotides

XSu(H)1 and *XSu(H)2* (GenBank accession number U60093 and U60094, respectively) including only open reading flame was amplified by polymerase chain reaction (PCR) with a *Xenopus* neurula library (a kind gift from Prof. D.A. Melton) using the following primer set;
XSu(H)1
5'-ATGGATCCATGCAACCTGGCATTCCT and
5'-TAACTCGAGTTAGGACACTACTGCTG;
XSu(H)2
5'-ATATCGATATGAAGTTTGGGGAGAGG and
5'-AATCTAGATTAGGGACACTACTGCTGC. Both of isolated XSu(H)1 and XSu(H)2 fragments were subcloned into pCS2+ vector (Turner and Wentraub, 1994) at *BamHI/XhoI* or *ClaI/XbaI* site, respectively. *XSu(H)2 DNA-binding mutant* (*XSu(H)2DBM*) was generated by PCR as a template of *XSu(H)1DBM/pCS2+* (Wettstein *et al*., 1997) and subcloned into pCS2+ vector at *BamHI/XhoI* site. *XESR-1* was generated by PCR with a *Xenopus* neurula library (a kind gift from Prof. D.A. Melton) using following primer set: 5'-ATGGATCCATGGCTCCTACCAGCATT and 5'-TAACTCGAGTCACCAGGGGCGCCATA and subcloned into pCS2+ vector at *BamHI/XhoI* site. *Dominant-negative form of XESR-1* (*DN XESR-1*), which was deleted the carboxyl-terminal WRPW motif (Fisher *et al*., 1996, Giebel and Campos-Ortega, 1997), was created by PCR as a template of *XESR-1/pCS2+* and subcloned at *BamHI/XhoI* site. *NICD/pCS2+* and *Xnr2/pCS2+* plasmids were kind gifts from Prof. C. Kintner (Wettstein *et al*., 1997) and Prof. J. C. Smith (Jones *et al*., 1995). The following morpholino antisense oligonucleotides (MO) were designed as suggested by the manufacturer (Gene Tools, LLC) for XSu(H)1 (5'-TGTATTTAGGAATGCCAGGTTGCAT) and for XSu(H)2 (5'-TCCCCAAACTTCATTCCGCTTCCCA). The standard morpholino provided by Gene Tools was used as the control morpholino.

RNA synthesis and microinjection

All capped mRNAs were synthesized from linearized plasmids using SP6 RNA polymerase (Epicentre Technology). Capped mRNA was made using mCAP RNA synthesis kit (Gibco BRL) according to the manufacturer's instructions. Fertilization, culture and microinjection were performed as described previously (Moon and Christian, 1989, Asashima *et al*., 1990). One blastomere of a two- or four-cell-stage embryo was injected with 5 nL mRNA or MO solution (see text and tables).

Whole-mount in situ *hybridization analysis*

Whole-mount *in situ* hybridization was performed according to the improved method of Shain and Zuber (Shain and Zuber, 1996). Hybridized probes were visualized according to the Röch Diagnostics DIG protocol, with the minor alternation that 0.45 μl NBT (75 mg/ml in dimethyl formamide) and 3.5 μl BCIP (Röch Diagnostics) were added to 1 ml AP buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgSO_4$, 0.1% Tween 20, 2.5 mM levamisole]. The antisense RNA probe of *Xbrachyury* (a kind gift from Prof. J. C. Smith) was prepared as described (Smith *et al*., 1991) by linearizing with *EcoRI* and transcribed with T7 RNA polymerase. β-galactosidase mRNA was produced from pCMV-SPORT β-gal (Stratagene).

RT-PCR analysis

Total RNA was extracted from embryos or animal caps using the Isogen solution (Nippongene). Oligo (dT)-primed first strand cDNA was prepared from 0.5 μg of total RNA using ReverscriptI (Wako, Japan). Each PCR was performed with this cDNA as a template. The RT-PCR program was 95˚C for 2 min, 55˚C for 2 min and 20-30 cycles of 72˚C 1 min, 95˚C 30 sec, 55˚C 30 sec. Several primer sequences are as follows;
XSu(H)1
upstream, 5'-GTTCAGAGCTCTTCTTTTTCTG-3' and
downstream, 5'- AGAACAATATGATGCCTTGGCT-3';
XSu(H)2
upstream, 5'-AAGCTGCGGAGTTAGGGAGA-3' and
downstream, 5'- TCAGCTGCTGCATTTCTTGC-3';
Histone H4
upstream, 5'-CGGGATAACATTCAGGGTATCACT-3' and
downstream, 5'-ATCCATGGCGGTAACTGTCTTCCT-3'; *Xbrachyury*
upstream, 5'-CGCTGGAAGTATGTGAATGG-3' and
downstream, 5'-TCATTCTGGTATGCGGTCAC-3';
MyoD
upstream, 5'-GACGACCCCTGTTTCAATAC-3' and
downstream, 5'-GGTTAGTTGAGGTGTATCGC-3';
Xvent1
upstream, 5'-TTCCCTTCAGCATGGTTCAAC-3' and
downstream, 5'-GCATCTCCTTGGCATATTTGG-3';
chordin
upstream, 5'-AACTGCCAGGACTGGATGGT-3' and
downstream, 5'-GGCAGGATTTAGAGTTGCTTC-3';
JNK1
upstream, 5'-CCAAGAGAGCTTATCGGGAAC-3' and
downstream, 5'-TCCCAAGATGACTTCTGGAGC-3';
goosecoid
upstream, 5'-ACAGCATACGATGGTGCA-3' and
downstream, 5'-ACTTCATGGTACTGCTGG-3';
X-ESR-1
upstream, 5'-ACAAGCAGGAACCCAATGTCA-3' and
downstream, 5'-GCCAGAGCTGATTGTTTGGA-3'. Negative control (-RT) was performed using the same program without reverse transcriptase. These cycle numbers located within the linearity of the growth curve prior to saturation.

Western blotting

Synthetic RNA of the myc-fused construct was injected into both blastomeres of a two-cell stage embryo, which was sampled at the gastrula stage (stage 10.5). XSu(H)1 and XSu(H)2 protein with 6myc-epitope tags were detected using anti-myc mouse monoclonal antibody, 9E10 (Santa Cruz Biotechnology) as the primary antibody and Alexa Fluor®680-conjugated anti-mouse IgG (H+L) antibody (Molecular Probes) as the secondary antibody. The fluorescent bands were detected by Odyssey ODY-9201-S (LICOR). As the internal control, actin bands were detected by staining with Coomassie brilliant blue (CBB).

Histology

The embryos were fixed with 4% paraformaldehyde for 12 hours at 4°C. They were then dehydrated through a graded series of methanol, cleared in xylene, embedded in TissuePrep (Fisher Scientific, U.S.A.) and sectioned serially at 10 μm. The sections were stained with hematoxylin and eosin.

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