Xenopus laevis POU91 protein, an Oct3/4 homologue, regulates competence transitions from mesoderm to neural cell fates

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Cellular competence is defined as a cell's ability to respond to signaling cues as a function of time. In *Xenopus laevis*, cellular responsiveness to fibroblast growth factor (FGF) changes during development. At blastula stages, FGF induces mesoderm, but at gastrula stages FGF regulates neuroectoderm formation. A *Xenopus* Oct3/4 homologue gene, *XLPOU91*, regulates mesoderm to neuroectoderm transitions. Ectopic *XLPOU91* expression in *Xenopus* embryos inhibits FGF induction of *Brachyury* (*Xbra*), eliminating mesoderm, whereas neural induction is unaffected. *XLPOU91* knockdown induces high levels of *Xbra* expression, with blastopore closure being delayed to later neurula stages. In morphant ectoderm explants, mesoderm responsiveness to FGF is extended from blastula to gastrula stages. The initial expression of mesoderm and endoderm markers is normal, but neural induction is abolished. *Churchill* (*chch*) and *Sip1*, two genes regulating neural competence, are not expressed in *XLPOU91* morphant embryos. Ectopic *Sip1* or *chch* expression rescues the morphant phenotype. Thus, *XLPOU91* epistatically lies upstream of *chch/Sip1* gene expression, regulating the competence transition that is critical for neural induction. In the absence of *XLPOU91* activity, the cues driving proper embryonic cell fates are lost.

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Introduction

During early embryonic development, cells must sense the dimension of time. Cells located in a given place and time respond to regional external signaling cues in a process called 'induction'. However, the same cells may respond differently to similar regional cues if found in a new time setting. This change in response to a signal as a function of time is called

'competence'. During early *Xenopus laevis* development, cells respond differentially to fibroblast growth factor (FGF) as time changes. In blastula stages, ectoderm is induced to mesoderm by proteins of the FGF family, which mimic signals secreted from the animal and vegetal poles to neighboring marginal zone cells (reviewed by Kimelman *et al*, 1992). By gastrula stage, ectoderm cells lose mesoderm-inductive competence (Jones and Woodland, 1987; Green *et al*, 1990) and are now induced to neural tissue by BMP antagonists that are secreted from the Spemann organizer during gastrulation (Harland and Gerhart, 1997). At this stage, FGF signaling caudalizes neural tissue to posterior fates such as midbrain, hindbrain and spinal cord (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Some other studies have shown that FGF signaling is also required, but not sufficient for general neural induction (Streit *et al*, 2000; Wilson *et al*, 2000). Thus, FGF and downstream MAP kinase signaling play at least two inductive roles at different stages of early development. Initially, FGF/MAP kinase signaling is required for proper mesoderm formation (LaBonne *et al*, 1995; Umbhauer *et al*, 1995), and at later stages, it is required for proper neural tissue formation. Little is known about the molecular components regulating these time-shift transitions.

Our previous studies show that one of the *X. laevis* Oct3/4 class V homologue genes, *XLPOU91*, regulates mesoderm formation in the developing embryo by modulating FGF competence timing during mesoderm induction (Henig *et al*, 1998). *XLPOU91* is zygotically expressed ubiquitously in all three germ layers at blastula to gastrula stages (Frank and Harland, 1992). It rises to peak expression levels during gastrula stages, the time when FGF competence shifts from mesodermal to neural fates. Ectopic *XLPOU91* protein expression inhibits mesoderm induction by FGF in whole embryos and animal cap (AC) ectoderm explants, without blocking neural induction (Henig *et al*, 1998). Embryos expressing ectopic *XLPOU91* protein levels resembled embryos having the FGF dominant-negative receptor phenotype (Amaya *et al*, 1991). *XLPOU91* perturbation of mesoderm formation was accomplished by antagonizing transcription of the FGF target gene, the mesodermal-specific *Xenopus* *Brachyury* (*Xbra*) transcription factor (Isaacs *et al*, 1994; Henig *et al*, 1998). Proper expression of *Xbra* is required for normal mesoderm development (Conlon *et al*, 1996). Although ectopic *XLPOU91* expression inhibited FGF activation of *Xbra* expression, it did not prevent FGF induction of the MAP kinase cascade in the same cells (Henig *et al*, 1998). Thus, although transcriptional activation of an FGF-mesodermal direct-target gene (*Xbra*) is inhibited by *XLPOU91*, the FGF signaling pathway is left intact, and it may later participate in neural induction and patterning. Antisense RNA injection inhibited translation of endogenous *XLPOU91* transcripts in AC explants; mesoderm competence to FGF was extended to

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gastrula stages in these explants (Henig *et al*, 1998). XLPOU91 reaches peak levels at gastrula stages, when it could act to suppress FGF activation of *Xbra* expression (Frank and Harland, 1992). These observations suggest that XLPOU91 protein is required for the proper temporal response of embryonic cells to FGF signaling.

Recently, two additional proteins were shown to regulate the FGF transition from mesodermal to neural competence. The Churchill (chch) zinc-finger protein regulates expression of the Smad interacting protein, Sip1 (Eisaki *et al*, 2000; van Grunsven *et al*, 2000; Papin *et al*, 2002; Sheng *et al*, 2003). Sip1 likely modifies Smad1/5 and Smad2/3 protein activities (Postigo, 2003) in a manner that sensitizes ectoderm to neural induction (Eisaki *et al*, 2000; Nitta *et al*, 2004), shifting competence from a mesodermal to neural direction (Sheng *et al*, 2003). In the absence of chch or Sip1 proteins, neural tissue formation is compromised in *Xenopus*, chick and mouse embryos (Higashi *et al*, 2002; Van de Putte *et al*, 2003; Sheng *et al*, 2003; Nitta *et al*, 2004). Mutations in the human Sip1 locus are associated with Hirschsprung disease-mental retardation syndrome; a similar phenotype was mimicked in Sip1 knockout mice (Van de Putte *et al*, 2003).

Using an *XLPOU91* antisense morpholino oligonucleotide (MO), we knocked down endogenous *XLPOU91* protein activity in *X. laevis* embryos. *XLPOU91* morphant embryos have impaired and delayed development. Morphant embryos over-express the *Xbra* gene, yet a large panel of early mesoderm and endoderm markers are expressed properly. Initial endoderm and mesoderm induction seems normal, whereas neural induction is abolished. Differentiation marker expression in all tissues is inhibited. Morphant embryos attempt gastrulation, but appear to have lost proper time cues. By late neurula stages, these embryos are not viable. Expression of the *chch* and *Sip1* genes is strongly reduced in morphant embryos. In a reciprocal manner, ectopic *XLPOU91* protein levels activate *chch* and *Sip1* expression in embryos. The *XLPOU91* morphant phenotype is rescued by ectopic expression of *chch* or *Sip1* proteins. Thus, the *Oct3/4* homologue, *XLPOU91*, lies at an important crossroad controlling cellular competence during early development. The absence of *XLPOU91* protein activity disrupts the expression of the crucial *chch/Sip1* genetic network that is required for the correct temporal and spatial regulation of cell fates.

Results

The *XLPOU91* MO effectively perturbs early *Xenopus* development

To knock down *XLPOU91* embryonic protein activity, we utilized an antisense MO designed to the 5' end of the *XLPOU91* gene (Materials and methods); a 5 bp mismatch *XLPOU91* MO (mm-MO) was designed to serve as a negative control. Two assays were used to determine if the MO disrupts endogenous *XLPOU91* protein activity. In the first, we injected *XLPOU91* MO into one-cell embryos, and blastula and early gastrula stage AC explants were removed and treated with FGF (Supplementary Figure S1). We scored expression of the *Brachyury* (*Xbra*) gene, expecting that the *XLPOU91* MO would extend the competence window of FGF-treated gastrula stage AC explants, as with *XLPOU91* antisense RNA (Henig *et al*, 1998). *Xbra* expression was indeed

Figure 1 The *XLPOU91* MO specifically blocks protein translation *in vivo*. (A) One-cell-stage embryos were injected in the animal hemisphere with 0.2–1.0 ng of RNA encoding the *XLPOU91*-Myc fusion protein (lanes 2–4) and 15 ng of *XLPOU91* MO (lanes 5–8). Protein was isolated from pools of 10 embryos at stage 10.5: uninjected control (lane 1), *XLPOU91*-Myc, 0.2–1.0 ng (lanes 2–4), *XLPOU91* MO injected (lane 5), *XLPOU91*-Myc 0.2–1.0 ng + *XLPOU91* MO (lanes 6–8). Western analysis was performed using the 9E10 Myc antibody. As a positive control, α -tubulin protein was detected. (B) One-cell-stage embryos were injected in the animal hemisphere with 0.8 ng of RNA encoding the *XLPOU91*-Myc protein (lanes 2–4) or 1.6 ng of RNA encoding the *XMeis3*-Myc protein (lanes 5–7) and 15 ng of *XLPOU91* MO (lanes 3 and 6) or *XLPOU91* mm-MO (lanes 4 and 7). Western blot analysis was performed on stage 11.5 extracts: uninjected control (lane 1), *XLPOU91*-Myc (lane 2), *XLPOU91*-Myc + MO (lane 3), *XLPOU91*-Myc + mm-MO (lane 4), *XMeis3*-Myc (lane 5), *XMeis3*-Myc + MO (lane 6), *XMeis3*-Myc + mm-MO (lane 7). Analysis was performed as described in (A).

activated in *XLPOU91* MO ACs removed at gastrula stages and treated with FGF in comparison to control ACs (Supplementary Figure S1). *Xbra* expression was also stimulated in *XLPOU91* MO ACs in the absence of FGF treatment (Supplementary Figure S1). This observation recapitulates our previous results using antisense *XLPOU91* RNA (Henig *et al*, 1998). In a second assay, to demonstrate that the MO was inhibiting *XLPOU91* protein translation *in vivo*, *XLPOU91*-Myc encoding mRNA was co-injected with the *XLPOU91* MO (Figure 1A). As determined by Western analysis of protein extracts isolated from gastrula embryos, the *XLPOU91* MO prevented translation of the *XLPOU91*-Myc protein in a dose-dependent manner (Figure 1A). As controls for MO specificity, we determined that the *XLPOU91* mm-MO cannot block *XLPOU91*-Myc translation (Figure 1B); in addition, translation of the *XMeis3*-Myc protein is not blocked by the wild-type *XLPOU91* MO *in vivo* (Figure 1B). Thus, the MO efficiently and specifically blocks *XLPOU91* mRNA translation *in vivo*.

Figure 2 *XLPOU91* MO slows blastopore closure in *Xenopus* embryos. Embryos at the one-cell stage were injected with 16 ng of *XLPOU91* MO or *XLPOU91* mm-MO. (A) Embryos are at stage 11.5, uninjected control (left panel), mm-MO injected (center panel) and MO injected (right panel). Phenotypes are analyzed at stage 12. In the control group ($n = 34$), 74% of the embryos are at stage 12–12.5, in the mm-MO group ($n = 32$), 72% of the embryos are at stage 12–12.5 and in the MO group ($n = 38$), 76% of the embryos are at stage 11–11.5; none of the embryos are at stage 12. On average, MO embryos lag about a stage behind. When control and mm-MO embryos reach stage 19, MO embryos are at stage 15–16. The mm-MO was compared to controls and MO in five independent experiments ($n = 292$ embryos). (B) Embryos are at stage 15, uninjected controls (upper panel) and MO-injected embryos (lower panel). (C) Embryos are at stage 20, uninjected control (top embryo, asterisk) and MO-injected embryos (lower six embryos). For the experiment shown in (B, C), the embryo phenotypes are summarized in Table 1. (D) Wild-type *XLPOU91* RNA rescues the morphant phenotype. One-cell-stage embryos were co-injected with 16 ng of *XLPOU91* MO and/or 1 ng of full-length *XLPOU91* encoding RNA. No difference was observed when the MO or RNA was co-injected in the same microinjection needle or in separate needles. Representative embryos are shown from gastrula to early tailbud stages. When 90% of the control embryos ($n = 48$) reached stage 11.5, 91% of the MO-injected embryos ($n = 35$) were at stage 10.5 and 85% of the *XLPOU91* + MO co-injected rescued embryos ($n = 65$) were at stage 11.5; the remaining 15% were at stage 11. At later stages, when controls reached stage 13 ($n = 43$), MO-injected embryos ($n = 27$) were at average stage 11.5 and the co-injected rescued embryos ($n = 60$) were at average stage 12.7.

XLPOU91 MO or *XLPOU91* mm-MO was injected into one-cell-stage embryos to determine embryonic phenotypes. *XLPOU91* mm-MO gastrula stage embryos had a normal phenotype as the uninjected controls (Figure 2A, left and center panels). Whereas the controls grew normally to mid-gastrula stages, the *XLPOU91* MO-injected embryos lagged behind, being at early gastrula stages (Figure 2A, compare right panel with left panel), when using blastopore size as the defining criterion. When controls arrived at mid-neurula stages (Figure 2B, top panel), the *XLPOU91* morphant embryos appeared to be at late gastrula stages (Figure 2B, bottom panel). In a representative experiment, when controls were at stage 14, the average stage of the morphant embryos was about stage 11.5 (Table I). In less extreme morphant phenotypes, *XLPOU91* morphant embryos could partially succeed in abnormally developing from gastrula stages, resembling more

advanced neurula-like stages (stages 16–17; Figure 2C, lower six embryos), whereas sibling controls developed normally until stage 20 (Figure 2C, asterisked top embryo). However, in more extreme phenotypes, by late neurula to early tailbud stages, embryo survival was low (not shown).

To demonstrate MO specificity for the *XLPOU91* protein, one-cell-stage embryos were co-injected with ectopic levels of wild-type *XLPOU91* encoding RNA and the MO. Co-injected embryos had a rescued phenotype, in comparison to morphant embryos. These embryos gastrulated and underwent typical elongations, having fairly normal morphology, with much higher survival levels than morphant embryos (Figure 2D). Ectopic expression of the *XLPOU91* protein can eliminate the morphant phenotype, suggesting that the observed phenotype is specific to the knockdown of endogenous *XLPOU91* protein activity.

Table 1 Phenotype distribution in XLPOU91 MO-injected embryos

Phenotype: average stage = 11.5		Number of embryos
1	Stage 10.5	19/61
2	Stage 11	11/61
3	Stage 11.5	5/61
4	Stage 12	2/61
5	Stage 12.5	17/61
6	Stage 13	6/61
7	Stage 14	1/61
Control embryos: average stage = 14		Number of embryos
1	Stage 13.5	7/29
2	Stage 14	15/29
3	Stage 15	7/29

XLPOU91 MO (12–16 ng) was injected at the one-cell stage. One representative experiment is shown. Uninjected embryos served as negative control. Similar results have been seen in over 50 independent experiments.

Initial induction of neural, but not endo-mesodermal markers, is inhibited in XLPOU91 morphant embryos

Embryos injected with the *XLPOU91* MO in the animal hemisphere at the one-cell stage were cultured to gastrula stages, and mesoderm marker expression was examined by semi-quantitative RT-PCR analysis. Gastrula stage expression of the *Xbra*, *XMyoD* and *gooseoid* (*gsc*) genes was increased in morphant embryos versus controls (Figure 3A, left panel). Apparently, knockdown of XLPOU91 protein levels changes the cell-type balance, shifting it towards mesoderm formation. This effect is more pronounced when one-cell-stage embryos are injected into presumptive ectoderm (animal pole) and not mesoderm (marginal zone) regions (not shown), supporting the idea that the lack of XLPOU91 activity could alter ectodermal cells to mesodermal fates, thus explaining the expression of *Xbra* in XLPOU91 morphant AC explants (Supplementary Figure S1). By whole-mount *in situ* hybridization, we observed high levels of *Xbra* expression spreading into the ectodermal animal pole regions of morphant embryos (Figure 3A, right panel). In gastrula and neurula stage morphant embryos, additional marker expression was examined. Despite the dramatic gastrulation phenotypes, a large number of early-expressed endo-mesodermal markers such as *XNR2-4-5-6*, *derriere*, *XHex*, *chordin*, *Mix1*, *Mixer*, *Edd*, *Xvent1* and *Xvent2* were expressed normally in early gastrula stage morphant embryos (Figures 3B and 4A).

The expression of proneural *Sox* and *Zic* genes was examined in gastrula stage morphant embryos. Expression of the *Sox2* gene, a key regulator of early neural plate specificity, is highly inhibited at mid-gastrula stages, as is expression of *Zic3* and *ZicR1* (Figures 4A and 6A); expression of these genes is also inhibited at mid- and late neurula stages (Figure 4B, C and E). One exception to this rule was *Sox3*, whose expression is normal in XLPOU91 morphant embryos at all stages examined (Figures 4A–C, E, 5B, E and 6A). In neurula stage embryos, expression of panneural markers such as *NCAM* and *nrp1* (Figures 4B–E, 5B, D and 6B) and anterior markers such as *otx2* and *XAG1* is inhibited (Figures 4B, C, E and 5D). Expression of posterior neural markers such as *HoxB9*, *Krox20* and *Engrailed2* and the neural crest marker *slug* is also highly reduced (Figure 4C). Expression of a later

Figure 3 The effects of XLPOU91 morphant phenotype on expression of mesoderm and endoderm markers in gastrula stage embryos. (A) One-cell-stage embryos were injected in the animal hemisphere with 12–16 ng of *XLPOU91* MO. Left panel: Total RNA was isolated from pools of five stage 11.5 embryos in each group. Semiquantitative RT-PCR analysis was performed with the markers *MyoD*, *Gsc* and *Xbra*. In all the experiments shown, *XHis4* served as a control for quantitating RNA levels in the different samples. As a control, RT-PCR was performed on total RNA isolated from normal embryos in all shown experiments. In this experiment, when control embryos reached stage 12–12.5 ($n = 50$), MO embryos ($n = 30$) were at stage 11. Right panel: Embryos were fixed at stage 11.5 and whole-mount *in situ* hybridization was performed with the *Xbra* probe. Embryos are oriented, animal pole right, vegetal pole left, in the control and MO-injected embryos. Note the marginal zone ring expression in the control ($n = 11$) versus the spreading into the animal pole region of the MO-injected embryo ($n = 11/13$). (B) One-cell-stage embryos were injected in the animal hemisphere with 15 ng of *XLPOU91* MO. Total RNA was isolated from pools of 10 stage 10.5 embryos. RT-PCR analysis was performed with the markers *XNR2*, *XNR4*, *XNR5*, *XNR6*, *derriere*, *chordin*, *XHex*, *Mix1*, *Mixer* and *Edd*. In this experiment, when controls reached stage 12–12.5 ($n = 52$), MO embryos ($n = 22$) were at stage 10.5–11.

mesodermal marker for muscle differentiation, *muscle actin*, is highly inhibited as is *XMyoD* at later neurula and early tailbud stages (Figures 4C–E, 5B and D). At later stages, expression of the ventral-specific *Xvent1* and *Xvent2* genes is not inhibited (Figure 4C), but expression of epidermal-specific *cytokeratin* is reduced (Figure 4C). Expression of the *Edd* endodermal marker, which is normal at early gastrula stages (Figure 3C), is highly inhibited at mid- and later neurula stages (Figure 4B and C). As with phenotypes, marker expression is normal in embryos expressing the XLPOU91 mm-MO (Figure 5B). Thus, whereas initial induction of endoderm and mesoderm appears to be normal or even slightly enhanced for many markers in morphant embryos, initial neural induction is highly inhibited. The ability of cells to undergo terminal differentiation to mesoderm, endoderm or epidermis tissue is also compromised in morphant embryos. As shown, XLPOU91 morphant embryos were morphologically rescued when co-injected with full-length *XLPOU91* mRNA (Figure 2D). Neural and mesodermal

Figure 4 Neural induction is inhibited in XLPOU91 morphant embryos. (A) One-cell-stage embryos were injected in the animal hemisphere with 15 ng of XLPOU91 MO. RNA was isolated from pools of eight gastrula stage 11.5 embryos. RT-PCR analysis was performed with the markers *Sox2*, *Sox3*, *ZicR1*, *Zic3*, *Xvent1* and *Xvent2*. (B) One-cell-stage embryos were injected in the animal hemisphere with 15 ng of XLPOU91 MO. Total RNA was isolated from pools of eight stage 14 embryos. RT-PCR analysis was performed with the markers *ZicR1*, *Zic3*, *Sox2*, *Sox3*, *Edd*, *XAG1*, *nrp1* and *NCAM*. In this experiment, when 97% of the controls reached stage 13–14 ($n = 34$), 83% of the MO embryos ($n = 18$) were at stage 10.5–11.5. (C) One-cell-stage embryos were injected in the animal hemisphere with 15 ng of XLPOU91 MO. Total RNA was isolated from pools of 10 neurula stage 17 embryos. RT-PCR analysis was performed with the markers *NCAM*, *nrp1*, *Sox3*, *Sox2*, *XAG1*, *OTX2*, *Eng2*, *Krox20*, *Hoxb9*, *Slug*, *epidermal cytokeratin*, *muscle actin*, *MyoD*, *Xvent1*, *Xvent2* and *Edd*. In this experiment, when controls reached stage 16–17 ($n = 45$), MO embryos ($n = 18$) were at stage 11. (D) Wild-type XLPOU91 RNA induces muscle and neural marker expression in rescued morphant embryos. One-cell-stage embryos were injected in the animal hemisphere with 15 ng of XLPOU91 MO and/or 1 ng of XLPOU91 encoding RNA. Total RNA was isolated from pools of eight early tailbud stage 21 embryos. RT-PCR analysis was performed with the markers *nrp1* and *muscle actin*. (E) Spemann organizer-mediated neural induction is inhibited in AC explants expressing the XLPOU91 MO. Early gastrula stage Spemann organizer (DMZ) explants were recombined with blastula stage control or XLPOU91 MO-injected (15 ng) AC explants. Explants and embryos were cultured to neurula stage 18 and total RNA was isolated from pools of 18 explants or seven embryos. RT-PCR analysis was performed with the markers *NCAM*, *XAG1*, *OTX2*, *Sox2*, *Sox3* and *muscle actin*. As a control, RT-PCR was performed on total RNA isolated from normal embryos (lane 1), control embryos (lane 2), morphant embryos (lane 3), control ACs (lane 4), morphant ACs (lane 5), control DMZ explants (lane 6), recombinant DMZ + control AC explants (lane 7) and recombinant DMZ + morphant AC explants (lane 8). In this experiment, when controls reached stage 13.5–14 ($n = 30$), MO embryos ($n = 40$) were at stage 11.

tissues undergo differentiation in these rescued embryos, as tailbud stage embryos re-expressed fairly normal levels of *nrp1* and *muscle actin* in comparison to the morphant embryos (Figure 4D).

In contrast to whole embryos, expression of cell differentiation markers is not inhibited in morphant explants induced at later gastrula stages. In early gastrulae, noggin induces ventrally fated mesoderm cells to dorsal–lateral muscle fates (Re'em-Kalma *et al*, 1995). In gastrula stage ventral marginal zone (VMZ) explants, noggin treatment induces expression of dorsal–lateral mesoderm and neural markers such as *muscle actin* and *nrp1* (Supplementary Figure S2; Bonstein *et al*, 1998). Interestingly, in morphant VMZ explants injected with noggin, there is no reduction in *muscle actin* or *nrp1* expression versus noggin-treated control VMZ explants (Supplementary Figure S2, compare lanes 4 and 6). However, endogenously regulated *muscle actin* and *nrp1* expression was strongly inhibited in morphant whole embryos (not shown) or morphant dorsal–lateral marginal zone explants in comparison to controls (compare lanes 7 and 8). Noggin dorsalization of ventral mesoderm to muscle in morphant explants is compatible with the activation of terminal differentiation markers. These results demonstrate that XLPOU91 morphant cells still maintain noggin-signaling responsiveness and the potential for expressing terminal differentiation

markers. However, in the embryo, the MO's disruption of timing events is so traumatic that endogenous tissues fail to differentiate

To further determine if the endogenous neural inducing signals were inhibited by the XLPOU91 protein knockdown, recombinant explants were made, in which 'naturally' neural inducing dorsal marginal zone (DMZ) Spemann organizer region explants were recombined with AC explants in the presence or absence of the XLPOU91 MO. In recombinant neural stage explants, the DMZ tissue induces *Sox2*, *NCAM*, *Otx2* and *XAG1* marker expression juxtaposed AC explants (Figure 4E, lanes 5–7). These inductions are strongly reduced in recombinant explants in which the AC was injected with the XLPOU91 MO (Figure 4E, compare lane 7 with lane 8). Expression of these identical genes is also reduced in morphant sibling embryos from the same experiment (Figure 4E, compare lane 2 with lane 3). Interestingly, *Sox3*, whose expression is not eliminated by the MO in whole embryos, is not highly induced in naïve AC ectoderm by the DMZ over background levels (Figure 4E, lanes 6–8).

XLPOU91 protein controls *chch* and *Sip1* mRNA levels

Recent studies have shown that the Churchill (*chch*) zinc-finger protein is required for the FGF competence transition of cells from mesodermal to neural fates (Sheng *et al*, 2003).

This function is mediated through *chch* transcriptional activation of the *Sip1* gene (Eisaki et al, 2000; van Grunsven et al, 2000; Papin et al, 2002; Sheng et al, 2003). Ectopic *XLPOU91* expression and knockdown phenotypes suggested that there might be a connection between *XLPOU91* protein and the *chch/Sip1* pathway. *XLPOU91* is expressed at the mid-blastula transition (MBT) (Frank and Harland, 1992) and therefore could epistatically lie upstream of *chch* and *Sip1* to regulate their expression. Ectopic *XLPOU91*, *chch* and *Sip1* levels all inhibit *Xbra* expression in *Xenopus* embryos and FGF-treated AC explants (Henig et al, 1998; Eisaki et al, 2000; van Grunsven et al, 2000; Papin et al, 2002; Sheng et al, 2003). Therefore, we examined *chch* and *Sip1* mRNA levels in

gastrula stage embryos either knocked down or overexpressing *XLPOU91* encoding RNA. In embryos overexpressing *XLPOU91* mRNA, *chch* and *Sip1* mRNA levels were sharply increased (Figure 5A, compare lanes 2 and 3). Reciprocally, *XLPOU91* morphant embryos expressed low levels of these genes (Figure 5A, compare lanes 5 and 6). In neurula stage morphant embryos rescued by coexpressing wild-type *XLPOU91* RNA (Figure 2D), there was a concomitant increase in *chch* and *Sip1* expression, in addition to rescued expression of the *muscle actin* and *nrp1* markers (Figure 5B). To determine a functional epistatic relationship, ectopic *chch* or *Sip1* encoding RNAs were separately co-injected along with the *XLPOU91* MO at the one-cell stage. Both of these RNAs rescue

Figure 5 XLPOU91 lies epistatically upstream of churchill and Sip1 proteins. (A) One-cell-stage embryos were injected in the animal hemisphere with 0.4 ng of *XLPOU91*-Myc encoding RNA (left panel, lanes 1-3) or 15 ng of *XLPOU91* MO (right panel, lanes 4-6). RNA was isolated from pools of eight gastrula stage 11.5 embryos. RT-PCR analysis was performed with the markers *churchill*, *Sip1* and *Xbra*. (B) Wild-type *XLPOU91* RNA activated *Sip1* and *chch* expression in rescued morphant embryos. One-cell-stage embryos were injected in the animal hemisphere with 15 ng of *XLPOU91* MO, *XLPOU91* mm-MO and/or 1 ng of *XLPOU91* encoding RNA. Total RNA was isolated from pools of eight neurula stage 18 embryos. RT-PCR analysis was performed with the markers *churchill*, *Sip1*, *Sox3*, *nrp1* and *muscle actin*. In this experiment, when controls reached stage 11-11.5 ($n=37$), the mm-MO embryos ($n=28$) were also at stage 11-11.5, MO embryos ($n=32$) were at stage 10.5 and the *XLPOU91* + MO-injected embryos ($n=39$) were also at stage 11-11.5. At later neurula stages, when controls reached stage 17-18 ($n=18$), the mm-MO embryos ($n=20$) were also at stage 17-18, MO embryos ($n=27$) were at stage 11 and 86% of the *XLPOU91* + MO-injected embryos ($n=29$) were also at stage 17-18. (C) Ectopic *Sip1* and *chch* RNA expression rescues the morphant phenotype. Embryos at the one-cell stage were co-injected with 16 ng of *XLPOU91* MO and/or full-length *Sip1* (0.5 ng) or *chch* (1 ng) encoding RNA. No difference was observed when the MO or specific RNA was co-injected in the same microinjection needle or in separate needles. Representative embryos are shown from gastrula to tailbud stages. In this experiment, when 88% of the controls were at stage 12 ($n=50$), 70% of the MO embryos ($n=40$) were at stage 10.5, 30% at stage 11, in *chch* + MO embryos, 66% ($n=64$) were at stage 12 and in *Sip1* + MO embryos ($n=56$), 71% were at stage 12. At later tailbud stages, when controls reached stage 23 ($n=35$), MO embryos ($n=23$) were at average stage 12.5, 71% of the *chch* + MO embryos ($n=35$) were at stage 23 and 82% of the *Sip1* + MO embryos ($n=28$) were at stage 23. Similar results were observed in five independent experiments, in which over 450 embryos were injected for each rescued group. (D) Ectopic *chch* expression rescues marker expression in morphant embryos. Embryos at the one-cell stage were co-injected with 15 ng of *XLPOU91* MO and/or 0.5-1 ng of full-length *chch* encoding RNA. Total RNA was isolated from pools of eight neurula stage 19 embryos. RT-PCR analysis was performed with the markers *muscle actin*, *XAG1*, *OTX2*, *nrp1*, *NCAM* and *Sip1*. In this experiment, when the controls were at stage 12-12.5 ($n=43$), the MO embryos ($n=30$) were at stage 10.5-11 and the *chch* + MO embryos ($n=56$) were at stage 12. At later stages, when the controls were at stage 18 ($n=17$), the MO embryos ($n=18$) were at stage 11.5-12 and the *chch* + MO embryos ($n=47$) were at stage 18. (E) *Chch* protein lies epistatically upstream of *Sip1* to rescue *XLPOU91* morphant embryo phenotypes. Embryos at the one-cell stage were co-injected with 16 ng of *XLPOU91* MO and/or full-length *Sip1* (0.5 ng) or *chch* (1 ng) encoding RNA. Total RNA was isolated from pools of eight gastrula stage 11.5 embryos. RT-PCR analysis was performed with the markers *Sip1*, *Sox2* and *Sox3*. Note the high levels of ectopically injected *chch* (lane 4) and *Sip1* (lane 5) RNA levels. Ectopic *chch* protein partially rescues *Sip1* and *Sox2* expression (compare lanes 2-4), whereas ectopic *Sip1* protein does not rescue *chch* expression, but does rescue *Sox2* expression (compare lanes 2, 3 and 5) in phenotypically rescued morphant embryos.

Figure 6 Sip1 neural induction is independent of XLPOU91 activity. (A) One-cell-stage embryos were injected in the animal hemisphere with 50 ng of *Sip1* MO (lane 3) and 0.8 ng of *XLPOU91-Myc* encoding RNA (lane 5) or 15 ng of *XLPOU91* MO (lane 4) and 0.8 ng of *Sip1* encoding RNA (lane 6). RNA was isolated from pools of eight gastrula stage 11.5 embryos. RT-PCR analysis was performed with the markers *Sox2*, *Sox3*, *Zic1* and *Zic3*. (B) Embryos from (A) were examined at neurula stage 18 (lanes 1–6). In parallel, blastula stage control (lane 7), *XLPOU91-Myc* (lane 8), *XLPOU91-Myc* + *Sip1* MO (lane 10), *Sip1* (lane 9) or *Sip1* + *XLPOU91* MO (lane 11) injected AC explants were cultured to neurula stage 18 and total RNA was isolated from pools of seven embryos or 18 explants. RT-PCR analysis was performed with the markers *NCAM* and *nrp*.

the XLPOU91 morphant phenotype when examined morphologically (Figure 5C). In gastrula stages, blastopore closure is quite normal (Figure 5C) in co-injected embryos. At late neurula–early tailbud stages, rescued embryos have elongated (Figure 5C, stage 20), and at tailbud stages when morphant embryos have died (not shown), the co-injected rescued embryos look completely normal, having a much higher frequency of survival (Figure 5C, stage 32) than morphant embryos. In experiments with strong rescues, embryos look remarkably normal (Figure 5C); in moderate rescues, embryos undergo greater elongation and survival into later neurula stages in comparison to morphant embryos (not shown). In both fully and partially *chch* rescued neural

stage embryos, there is a large increase in neural marker and *muscle actin* expression at neurula stages (Figure 5D); thus, cells are apparently differentiating. Similar results were seen in *Sip1* rescued embryos (not shown). Supporting the previous models for *chch*/*Sip* activities, ectopic *chch* mRNA microinjection induced *Sip1* and *Sox2* expression in the rescued gastrula stage morphant embryos (Figure 5E, compare lane 3 with lane 4). In contrast, ectopic *Sip1* mRNA microinjection, while efficiently rescuing phenotypes and *Sox2* expression, did not induce *chch* expression (Figure 5E, compare lane 3 with lane 5).

We used the *Sip1* MO (Nitta *et al*, 2004) to address *XLPOU91*/*Sip1* epistasis. Ectopic expression of the *Sip1* protein rescues gastrula and neurula stage neural marker expression in *XLPOU91* morphant embryos (Figure 6A and B, compare lanes 4 and 6), whereas ectopic expression of *XLPOU91* protein could not rescue neural marker expression in the *Sip1* morphant phenotype (Figure 6A and B, compare lanes 3 and 5). Extending these results, ectopic *XLPOU91* expression, like ectopic *Sip1* expression (Nitta *et al*, 2004), induces neural marker expression in AC explants (Figure 6B, compare lanes 7–9). *XLPOU91* neural induction is *Sip1* dependent, as co-injection of *XLPOU91* protein with the *Sip1* MO prevented neural induction in ACs (Figure 6B, compare lanes 8 and 10). In contrast, *Sip1* neural induction is not *XLPOU91* dependent, as co-injection of *Sip1* protein with *XLPOU91* MO did not inhibit neural induction (Figure 6B, compare lanes 9 and 11). Confirming this epistatic relationship, we also observed that *XLPOU91* induction of *chch* and *Sip1* expression in ACs is highly FGF-dependent (not shown). In AC explants lacking FGF signaling, *XLPOU91* neural induction is completely inhibited, whereas *Sip1* neural induction is nearly normal (not shown).

These results suggest that *XLPOU91* controls mesodermal to neural cell transitions by regulating the early FGF-dependent expression of the *chch* gene, which subsequently activates *Sip1* gene expression, thus positioning this *Xenopus* Oct3/4 homologue protein, *XLPOU91*, epistatically upstream of this important *chch*/*Sip1* genetic pathway.

Discussion

Our previous studies showed that ectopic overexpression of *XLPOU91* protein mimicked the FGF inhibitory/*Xbra* knock-out phenotype (Amaya *et al*, 1991; Conlon *et al*, 1996; Henig *et al*, 1998). These embryos lost dorsal–posterior mesoderm tissues such as notochord and muscle, while continuing to express neural markers. Ectopic levels of *XLPOU91* protein inhibited *Xbra* expression in embryos and in FGF-treated AC explants (Henig *et al*, 1998). We also showed that expression of *XLPOU91* antisense RNA shifted mesoderm competence in FGF-treated AC explants. FGF continued to induce *Xbra* expression in ACs treated at gastrula stages, the time when normal competence responsiveness was lost. In the present study, we knocked down *XLPOU91* activity using an MO. The MO appears to inhibit endogenous *XLPOU91* protein levels. The *XLPOU91* MO prevents *in vivo* translation of an injected *XLPOU91-Myc* mRNA; ectopically expressed wild-type *XLPOU91* protein rescues the morphant phenotype, and injection of a 5 bp mismatch MO does not alter development. Morphant embryos undergo slower gastrulation, and blastopore closure occurs at later neurula stages. Morphant

embryos typically express high levels of *Xbra*, whereas meso-endodermal gene expression at gastrula stages is fairly normal or slightly enhanced for most markers. There is no difference between anterior, posterior or endo-mesodermal markers in this regard. However at later stages, markers of mesoderm, endoderm and epidermis differentiation are highly inhibited. This may not be surprising, as morphant embryo development is so perturbed, likely preventing correct differentiation of all germ layer tissues. In contrast to mesoderm and endoderm, neural tissue is not initially induced in morphant embryos or explants. The earliest expressed proneural markers are not expressed in gastrula-neurula stage embryos or in AC explants recombined with inducing Spemann organizer tissue. The exception to this rule is the *Sox3* gene. Although the loss of XLPOU91 protein enables fairly normal expression of endo-mesodermal markers, neural induction is compromised. This observation suggests that when the responsiveness to FGF competence in the mesoderm is extended to gastrula stages, one consequence is the loss of neural induction. Furthermore, when the embryo lacks proper time cues, cells lose the ability to follow the endogenous blueprint to differentiate in the proper temporal and spatial manner. This loss of FGF mesoderm responsiveness is a necessary permissive event enabling neural induction and subsequent differentiation of embryonic cells *in vivo*.

We also investigated potential downstream targets of XLPOU91 activity. Recent studies showed that the zinc-finger protein Churchill (*chch*) is a key regulator of FGF competence; *chch* is a late FGF response gene required for neural induction in vertebrate embryos (Sheng *et al*, 2003). Like XLPOU91, *chch* overexpression blocks *Xbra* expression and mesoderm formation (Sheng *et al*, 2003). In AC explants, we also show that XLPOU91 cannot induce *chch* expression in the absence of FGF signaling. Chch protein appears to function by activating the transcription of another crucial protein, Sip1. Sip1 likely modifies Smad1/5 and Smad2/3 protein activities (Verschueren *et al*, 1999; Postigo, 2003). Fitting this model, our results show that XLPOU91 neuralizing activity requires FGF signaling, whereas Sip1 neuralizing activity does not. Sip1 modification of BMP signaling likely directs competence from a mesodermal to neural direction (Sheng *et al*, 2003), thus sensitizing ectoderm to neural induction (Eisaki *et al*, 2000). Ectopic Sip1 protein expression in embryos also directly suppresses *Xbra* expression causing the same gain-of-function phenotype as *chch* and XLPOU91 proteins (Papin *et al*, 2002). Similar to XLPOU91 morphant embryos, in Sip1 knockout mice, *Sox2* expression is reduced, whereas *Sox3* expression is normal (Van de Putte *et al*, 2003). In chick node graft assays, expression of the *Sox3* proneural gene is induced very early (1–2 h) in an FGF-dependent manner. In this same assay, *Sox2* expression takes much longer (9 h). However, later neuralizing signals are still required to stabilize *Sox3* expression and lock neural cell fates in ectoderm cells (Streit *et al*, 1998). *Sox3* expression is normal in Sip1, and XLPOU91 knockdown/knockout embryos, whereas *Sox2* expression is eliminated. Interestingly, *Sox3* was not induced by the Spemann organizer in our recombinant AC explant assay. Thus, the organizer signal that requires an FGF competence transition in naïve ectoderm, in order to induce neural tissue, likely acts independently of the pathway inducing *Sox3* expression. An earlier

signaling pathway (perhaps FGF) may act concomitantly with the organizer to induce *Sox3* expression. In addition, Sip1 specifically modulates BMP activity in neural but not mesodermal tissue, as Sip1 induces neural tissue in embryos and explants (Eisaki *et al*, 2000). In a similar manner, XLPOU91 morphant embryos express dorso-anterior endo-mesodermal markers induced by BMP antagonism, yet neural induction inhibition suggests that some specific aspects of BMP antagonism in the neural induction process are modified in the absence of XLPOU91 protein activity.

Because of the similarities between the ectopic and knock-down XLPOU91/*chch*/Sip1 phenotypes, we examined the epistatic relationship between these proteins. In XLPOU91 morphant embryos, *chch* and Sip1 expression was strongly reduced. Ectopic XLPOU91 protein expression significantly raised *chch* and Sip1 mRNA levels in XLPOU91 morphant and normal embryos. These results suggest that XLPOU91 lies upstream of *chch*/Sip1 gene expression. To address pathway epistasis, XLPOU91 morphant embryos were co-injected with mRNA encoding either full-length *chch* or Sip1 proteins. In co-injected embryos, we convincingly saw phenotypic rescue, at both morphological and marker expression levels. In *chch* rescued embryos, Sip1 mRNA levels were increased; ectopic Sip1 expression rescued XLPOU91 morphant phenotypes, but did not alter *chch* mRNA levels, supporting experimental observations suggesting that Sip1 lies downstream of *chch* protein (Sheng *et al*, 2003). The reciprocal observation was not observed: XLPOU91 protein could not rescue the Sip1 morphant phenotype in embryos or explants.

Chch was shown to be a late FGF response gene in the neural ectoderm of chick embryos (Sheng *et al*, 2003); so it is tempting to speculate that perhaps the extension of FGF responsiveness in the mesoderm of XLPOU91 morphant embryos prevents the proper temporal induction of *chch* expression in the neural ectoderm by FGF. By knocking down XLPOU91 protein activity, the extension of FGF-mesoderm competence and signaling induces higher and ectopic *Xbra* expression levels in the embryo. Morphant AC explants express *Xbra* in contrast to control ACs (Supplementary Figure S1A). We also observed that the increased expression of *Xbra* in morphant embryos is mainly in the ectodermal animal hemisphere regions. Ectopic *Xbra* expression was shown to disrupt gastrulation and spread mesoderm formation into AC ectoderm cells (Cunliffe and Smith, 1992). In the XLPOU91 morphant scenario, FGF competence disruption triggers incorrect temporal and spatial regulation of *Xbra* expression. Under these conditions, an early regulator of neural induction, such as *chch*, is not properly induced by FGF signaling because cells are 'stuck' in an untimely confused mesoderm responsiveness state. In the absence of XLPOU91 protein activity, the embryo loses the normal time cues required for proper cell fate identity.

XLPOU91 is one of three *X. laevis* Oct3/4 POU domain class V homologue genes that have been identified. Little is known about the functions of these genes. XLPOU91 is initially expressed at the onset of zygotic transcription at MBT. XLPOU/OCT-25 (Hinkley *et al*, 1992), like XLPOU91, is also expressed zygotically at MBT and ubiquitously during a narrow expression window. Ectopic expression of XLPOU25 was shown to activate expression of *Xvent-2b*, which is a downstream target gene of BMP signaling (Cao *et al*, 2004). BMP ventralizing activity is mediated by downstream *vent1*

or *vent2* homeobox gene expression (Gawantka *et al*, 1995). *XLPOU91* and *XLPOU25* do not appear to be functionally redundant. Unlike *XLPOU91*, ectopic *XLPOU25* protein expression inhibits neural induction in addition to mesoderm induction (Cao *et al*, 2004). Interestingly, slightly increased *vent1/vent2* gene expression was observed in *XLPOU91* morphant embryos and explants (not shown). We additionally found that *XLPOU25* expression was increased and extended to later neurula stages in *XLPOU91* morphant embryos (not shown). *XLPOU91* protein could act directly to restrict the timing of *XLPOU25* gene expression, or perhaps general disruption of normal time cues in morphant embryos simply prevents the precise temporal downregulation of *XLPOU25* expression. These results suggest that *XLPOU91* and *XLPOU25* proteins could act in an antagonistic feedback manner to regulate early cell fates. Future experiments should clarify this point.

In Zebrafish embryos, the *pou2* class V POU gene is maternally expressed. In *pou2* morphant fish, gastrulation is delayed and cells stop dividing (Burgess *et al*, 2002). In *pou2* mutant embryos, FGF8 competence in the developing nervous system is lost and the mid-hindbrain border and hindbrain primordium regions of the embryo do not form properly (Burgess *et al*, 2002; Hauptmann *et al*, 2002; Reim and Brand, 2002). Zebrafish *pou2* protein is also required for proper endoderm formation (Lunde *et al*, 2004; Reim *et al*, 2004). These results suggest that early class V *Oct3/4* orthologue genes may have similar, but not necessarily identical, roles in early vertebrate development.

Oct3/4 is one of the earliest detected mammalian transcription factors. *Oct3/4* is expressed in pre-implantation embryos, and from the eight-cell stage, expression is high until morula stages, when expression is downregulated in the trophoblast and further maintained in the blastocyst inner cell mass (ICM). By E8.5, *Oct3/4* transcripts are detected only in germ cell progenitors (Rosner *et al*, 1990; Scholer *et al*, 1990). *Oct3/4* is expressed in pluripotential cells of the ICM and its expression is required for self-renewal and pluripotency in embryonic stem cells (Niwa *et al*, 2000). In *Oct3/4*-deficient embryos, pluripotent embryo cells are lost and it is thought that these cells will give rise to tissue-specific stem cells in the embryo (Nichols *et al*, 1998). Interestingly, *Oct3/4* activity was shown to enhance and promote ES cell differentiation of *Sox2*-expressing neuroectoderm-like cells (Shimozaki *et al*, 2003), perhaps analogous to *XLPOU91*. Although it is clear that *Oct3/4* protein is a key regulator in maintaining stem cell pluripotency, a definitive role for this protein in mammalian embryonic development is still somewhat enigmatic.

Is *XLPOU91* the *Oct3/4* orthologue in amphibians? *XLPOU91* plays a key role in early events regulating the ability of cells to define time and thus spatial cues in the early embryo. Most likely the amphibian embryo utilizes these three distinct *Oct3/4* homologues to perform parallel or perhaps different interacting functions that enable early embryonic cells to sense their environment. By regulating expression of the *chch/Sip1* proteins, *XLPOU91* protein regulates precise embryonic competence shifts, thus controlling cell differentiation and morphogenesis decisions in the early embryo.

Wnt and FGF signaling are required for vertebrate neural induction *in vivo* (Baker *et al*, 1999; Streit *et al*, 2000). One

could speculate that nearly all 'roads' of neural induction lead to BMP antagonism in the embryo. Early canonical Wnt signaling inhibits BMP transcription in presumptive neural ectoderm (Baker *et al*, 1999); FGF signaling mediates *Sip1* activation by converting *Smad1/5* transcriptional activators to repressors (Sheng *et al*, 2003), whereas secreted BMP antagonists expressed in the organizer directly block BMP ligand activity. Experimental evidence suggests that only when all three of these pathways operate simultaneously in the embryo, correct neural induction occurs (Baker *et al*, 1999; Streit *et al*, 2000; Sheng *et al*, 2003; Khokha *et al*, 2005). Thus, the overall goal of optimal BMP antagonism in the neuroectoderm is accomplished at a number of molecular levels during early development. This model could explain why *Xenopus* explants treated with ectopically high levels of a BMP antagonist (noggin) can still induce neural tissue when FGF signaling (Ribisi *et al*, 2000) or *XLPOU91* protein (Supplementary Figure S2) or *Sip1* protein (not shown) is knocked down, or in contrast, why ectopic Wnt, *XLPOU91* or *Sip1* protein expression induces neural tissue in ACs in the absence of BMP antagonist proteins (Baker *et al*, 1999; Eisaki *et al*, 2000; Figure 6). As long as the overall BMP signaling output is inhibited above a certain threshold in the cultured AC explant, it is probably inconsequential how this is accomplished. Thus, the contributory combination of each of these individual pathways *in vivo* concomitantly dictates the overall optimal levels of BMP antagonism essential for neural induction in embryonic neuroectoderm tissue.

Materials and methods

Xenopus embryos, explants and inducing factors

Ovulation, *in vitro* fertilization and embryo and explant culture were performed (Re'em-Kalma *et al*, 1995; Dibner *et al*, 2001). Embryos were staged according to Nieuwkoop and Faber (1967). AC explants removed at blastula or gastrula stages were treated with bFGF (150 ng/ml) (Henig *et al*, 1998). Explants were cultured in FGF to later gastrula stages; total RNA was isolated for RT-PCR analysis. In recombinant explants, Spemann organizer, DMZ pieces (45–60° in size), was removed at stage 10.25. *XLPOU91* MO-injected or control AC explants were removed at blastula stages. Recombinant explants were juxtaposed with watchmaker's forceps and cultured in 1 × Steinberg's medium to neurula stages (Bonstein *et al*, 1998).

RNA and morpholino injections

Capped *in vitro* transcribed full-length mRNA, *XLPOU91* (Henig *et al*, 1998), *Xenopus churchill* (Sheng *et al*, 2003), *Xenopus noggin* (Ribisi *et al*, 2000) and *Xenopus Sip1* (Eisaki *et al*, 2000) were injected into the animal hemisphere of one-cell-stage embryos. An antisense MO complementing the 5' region of the *XLPOU91* mRNA (Frank and Harland, 1992) was purchased from Gene Tools (Philomath, OR, USA). The sequence was 5'-CGGGTTGTGGGTAAAGGAAGGTAG-3'. As a negative control, a 5bp mismatch *XLPOU91* MO was also designed. The sequence was 5'-CGCtGtAGTGGcTAAAGGAAGGAG-3'. One-cell-stage embryos were injected with 15–20 ng in a 7.5–10 nl volume.

RT-PCR analysis

RT-PCR was performed (Wilson and Melton, 1994), except that random hexamers (100 ng/reaction) were used for reverse transcription. Primers used for PCR were *XHis4* (Gawantka *et al*, 1995), *Krox20*, *HoxB9*, *Engrailed2* and *NCAM* (Hemmati-Brivanlou and Melton, 1994), *otx2*, *Xbra*, *XAG1*, *Xvent1*, *Xvent2*, *gsc*, *muscle actin*, *epidermal cytokeratin*, *nrp1*, *slug*, and *chordin* (Harland lab database), *Sox2*, *Sox3*, *ZicR1* and *zic3* (Kato *et al*, 1999), *Xnr2*, *Xnr4* and *derriere* (Takahashi *et al*, 2000), *Xnr5* and *Xnr6* (Yang *et al*, 2002), *XHex* and *Mix1* (Chang and Hemmati-Brivanlou, 2000), *MyoD* (Nicolas *et al*, 1998), *Edd* (Sasai *et al*, 1996), *Sip1* (Eisaki

et al, 2000) and *chch*: U: 5'-ATGTGCGGAGGCTGCGTC-3', D:5'-CGTG GGTACATCGGGTAGAAC-3' (316 bp length).

Western blot analysis

Western blot analysis was performed (Dibner *et al*, 2001). For the XLPOU91-Myc vector, a full-length XLPOU91 PFU generated fragment was subcloned 5' to the Myc fusion site in the pCS2 + MT vector. The plasmid was linearized with *NotI* and transcribed with Sp6 to generate RNA encoding XLPOU91-Myc fusion protein. XLPOU91-Myc RNA was co-injected with 16 ng of XLPOU91 MO or XLPOU91 mm-MO into one-cell-stage embryos. Protein was isolated from pools of 10 embryos per group at gastrula stages. Total protein (30–50 µg) was typically loaded for electrophoresis. Western blot

analysis was performed using the 9E10 Myc antibody. As a control for protein loading, α -tubulin protein levels were determined in each sample.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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