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The dual regulator Sufu integrates Hedgehog and Wnt signals in the early *Xenopus* embryo

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ABSTRACT

Hedgehog (Hh) and Wnt proteins are important signals implicated in several aspects of embryonic development, including the early development of the central nervous system. We found that *Xenopus* Suppressor-of-fused (XSufu) affects neural induction and patterning by regulating the Hh/Gli and Wnt/β-catenin pathways. Microinjection of *XSufu* mRNA induced expansion of the epidermis at the expense of neural plate tissue and caused enlargement of the eyes. An antisense morpholino oligonucleotide against XSufu had the opposite effect. Interestingly, both gain- and loss-of-function experiments resulted in a posterior shift of brain markers, suggesting a biphasic effect of XSufu on anteroposterior patterning. XSufu blocked early Wnt/β-catenin signaling, as indicated by the suppression of *XWnt8*-induced secondary axis formation in mRNA-injected embryos, and activation of Wnt target genes in *XSufu*-MO-injected ectodermal explants. We show that XSufu binds to XGli1 and Xβ-catenin. In *Xenopus* embryos and mouse embryonic fibroblasts, Gli1 inhibits Wnt signaling under overexpression of β-catenin, whereas β-catenin stimulates Hh signaling under overexpression of Gli1. Notably, endogenous Sufu is critically involved in this crosstalk. The results suggest that XSufu may act as a common regulator of Hh and Wnt signaling and contribute to intertwining the two pathways.

Introduction

An important question in developmental biology is how multiple signaling pathways such as those activated by Hedgehog (Hh) and Wnt proteins are integrated to generate positional identity in the embryo. Originally identified as factors affecting *Drosophila* embryogenesis, the Hh and Wnt pathways are major signaling systems during animal development, stemness and cancer (Jiang and Hui, 2008; MacDonald et al., 2009). Binding of the Hh ligand to its receptor, Patched (Ptc), alleviates an inhibition from Ptc on a downstream membrane protein, Smoothened, which ultimately activates target genes through the Gli family of zinc-finger transcription factors. Vertebrate Gli1 is mainly a transcriptional activator, whereas Gli2 and Gli3 can act both as transcriptional activators and repressors, depending on their posttranslational modification (Jiang and Hui, 2008; Koebernick and Pieler, 2002; Ruiz i Altaba et al., 2007). A central theme of the Wnt pathway is to stabilize the transcription co-activator β-catenin. Stabilized β-catenin then accumulates in the nucleus and interacts with the T cell factor (TCF)/lymphoid enhancer factor (LEF) family of DNA binding transcription factors to promote expression of target genes (Angers and Moon, 2009; MacDonald et al., 2009).

In vertebrates, the developing neural tube and telencephalon are patterned along the dorsoventral axis by opposing actions of two signaling centers with Sonic hedgehog through activation of Gli transcription factors inducing ventral cell fates and Wnt signals *via* the transcriptional co-activator β-catenin inducing dorsal identities (Danesin et al., 2009; Lee et al., 1997; Saint-Jeannet et al., 1997). Studies in *Xenopus* embryos first showed that the anteroposterior polarity of the neural tube is determined by a gradient of Wnt/β-catenin signaling (Kiecker and Niehrs, 2001). A key role for posterior Wnt signals and anterior Wnt inhibition has now been validated in most animals (Niehrs, 2010; Petersen and Reddien, 2009). Overexpression of Hh ligands can stimulate anterior neural induction in *Xenopus* embryos (Franco et al., 1999; Lai et al., 1995), but the signaling mechanism and whether Hh signals are required in this process are not known. The precise role of Wnt signals in neural induction remains a matter of debate. While earlier studies suggested that maternal Wnt/β-catenin signals induce neural fate through inhibiting *BMP4* transcription (Baker et al., 1999) and promoting the expression of secreted BMP antagonists (Wessely et al., 2001), a more recent report challenged the view of Wnts as pro-neural inducers and suggested instead that neural induction requires inhibition of Wnt/β-catenin signaling (Heeg-Truesdell and LaBonne, 2006). A possible mechanism for the anti-neural activity of Wnt signals was proposed, by which Wnts through inhibition of

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Glycogen synthase kinase 3 (GSK3) prevent phosphorylation and proteosome-mediated degradation of the BMP transducer Smad1 (Fuentealba et al., 2007). An interesting and yet unresolved question is whether Hh and Wnt signaling interconnect and whether a common regulatory mechanism may exist that integrates the two pathways in neural induction and patterning.

Suppressor-of-fused (Sufu) is an intracellular inhibitor of Hh signaling whose impact varies from being only marginally important in *Drosophila*, where it was first described (Préat, 1992), to being an absolutely necessary regulator of this pathway in mammals (Cheng and Yue, 2008). Elimination of Sufu in the mouse leads to ligand-independent activation of the Hh pathway, and *Sufu*-homozygous mutant embryos die at mid-gestation with a ventralized spinal cord (Cooper et al., 2005; Svärd et al., 2006; Varjosalo et al., 2006). Mutations of *Sufu* are linked to medulloblastoma in human (Taylor et al., 2002; 2004) and an elevated risk for the same type of brain cancer in mice (Lee et al., 2007), suggesting that Sufu is a tumor suppressor gene. Sufu directly binds to the Gli proteins and is thought to antagonize their activity through distinct mechanisms: sequestering Gli proteins in the cytoplasm or inhibiting Gli transcriptional activity in the nucleus (Barnfield et al., 2005; Cheng and Bishop, 2002; Ding et al., 1999; Kogerman et al., 1999; Merchant et al., 2004; Murone et al., 2000; Paces-Fessy et al., 2004; Stone et al., 1999). In the resting state of Hh signaling, primary cilia are not required for Sufu to inhibit Gli proteins (Chen et al., 2009; Jia et al., 2009; Zeng et al., 2010). Activation of the pathway leads to the recruitment of Sufu-Gli complexes to cilia and triggers their rapid dissociation to allow Gli activation (Humke et al., 2010; Tukachinsky et al., 2010). On the other hand, an *in vitro* study showed that overexpressed murine Sufu can bind to β-catenin, export it from the nucleus and thereby negatively regulate β-catenin-dependent transcription (Meng et al., 2001; Taylor et al., 2004). A repressor form of Gli3 (Gli3R), which is generated in the absence of Hh signaling, can physically interact with and inhibit β-catenin, suggesting that a complex between β-catenin, Gli3R and Sufu may inhibit canonical Wnt signaling (Ulloa et al., 2007). Node defects in Sufu-depleted mouse embryos have been interpreted due to possible upregulation of β-catenin signaling (Cooper et al., 2005), but increased Wnt pathway activity has not been detected upon RNAi-induced loss of *Sufu* expression or in *Sufu* mutants (Svärd et al., 2006; Varjosalo et al., 2006). Thus loss-of-function data so far support a role of Sufu only as a Hh/Gli inhibitor, and a function of Sufu as an *in vivo* regulator of Wnt/β-catenin signaling remains to be established.

In this study, we compare Hh and Wnt signals during neural induction, anteroposterior patterning and specification of the eye field. We analyze the function of Sufu in the early *Xenopus* embryo and study its interaction with Hh/Gli and Wnt/β-catenin signaling. We further investigate the crosstalk between Gli1 and β-catenin signals and the role that Sufu plays therein. The experimental approach is based on the analysis of overexpressed proteins and depletion of Sufu in *Xenopus* embryos and cultured cells. Our results suggest that Sufu acts as a common inhibitor of Hh/Gli1 and Wnt/β-catenin signals and is required for the integration of the two pathways.

Results

Hh and Wnt signals regulate neural induction, anteroposterior patterning and eye development

We set out to compare Hh and Wnt signaling in the early *Xenopus* embryo, focusing on the formation of the neural plate, anteroposterior patterning and specification of the eye field (Fig. 1). Hh signals can promote the induction of neural markers (Franco et al., 1999; Lai et al., 1995), but whether they are essential for neural induction has not been addressed yet. Previous work on the contribution of Wnt signals to neural induction has been conflicting with some reports supporting pro-neural activity (Baker et al., 1999; Sokol et al., 1995; Wessely et al., 2001) and other studies suggesting that the Wnt pathway may counteract neural induction (Glinka et al., 1998; Heeg-Truesdell and LaBonne, 2006; Itoh et al., 1995). We found that injection of *XGli1* or *XBhh* mRNA into the animal pole of one blastomere at the 4-cell stage caused an expansion of the neural plate marker *Sox2* and a concomitant retraction of the epidermal marker *Cytokeratin* (Fig. 1B,B′; see Fig. S1A,A′ in the Supplementary material). In contrast, inhibition of Hh signaling by mRNA encoding a carboxyterminally truncated Gli3 protein (*Gli3C′ΔClaI*; Ruiz i Altaba, 1999) or a dominant-negative form of the Hh receptor Patched1 (*XPtc1ΔLoop2*; Koebernick et al., 2003) promoted epidermal development at the expense of neural tissue (Fig. 1C,C′; see Fig. S1B,B′ in the Supplementary material), suggesting an important function of Hh signals in the induction of neural tissue. Microinjection of a CMV promoter plasmid containing *XWnt3a* DNA that is expressed at the onset of zygotic transcription at mid-blastula stage depleted the neural plate and expanded the epidermis primarily in anterior territories of the embryo (Fig. 1D,D′). Injection of mRNA encoding a hormone-inducible Tcf (*THVGR*; Wu et al., 2005) followed by addition of Dexamethasone at stage 10 caused downregulation of *Sox2* expression (see Fig. S2A′ in the Supplementary material) suggesting that neural plate formation can be abrogated by Wnt signaling at the onset of gastrulation. On the contrary, inhibition of Wnt signaling by *dnXWnt8* mRNA (Hoppler et al., 1996) had the opposite effect and supported neural at the expense of epidermal fate (Fig. 1E,E′). Together, these data suggest that Hh signals through activation of the transcription factor Gli1 have a positive role in the induction of neural tissue, whereas zygotic Wnt/β-catenin signals exert a negative function in neural plate formation (Fig. 1F).

Overexpression of Hh ligands can induce anterior markers in neuralized explants (Lai et al., 1995) and affect anteroposterior hindbrain patterning *in vivo* (Franco et al., 1999). Wnt signals repress anterior cell fates and induce posterior neural development (Christian and Moon, 1993; Glinka et al., 1998; Kiecker and Niehrs, 2001; McGrew et al., 1995), but temporal aspects of Wnt signaling in neural patterning have not been thoroughly addressed yet. We observed that *XGli1* or *XBhh* mRNA induced posteriorward displacement of the brain markers *Otx2* (forebrain and anterior midbrain), *En2* (posterior midbrain), and *Krox20* (hindbrain rhombomeres 3 and 5) in injected embryos (Fig. 1H–H″; see Fig. S1C–C″ in the Supplementary material). On the other hand, *Gli3C′ΔClaI* or *XPtc1ΔLoop2* mRNA caused a very subtle anteriorward shift of these markers (Fig. I–I″; see Fig. S1D–D″ in the Supplementary material), suggesting that Hh/Gli signals although potent may only be of minor importance for anterior neural development. *XWnt3a* DNA led to an anteriorward shift of *Otx2* and an anterior expansion of *En2* expression, but consistent with a previous study (Saint-Jeannet et al., 1997) had no effect on *Krox20* expression within the hindbrain (Fig. 1J–J″). THVGR induced an anteriorward shift of *Krox20* when stimulated with Dexamethasone at stage 10 but had only a moderate effect upon activation at stage 14 (see Fig. S2B′,C′ in the Supplementary material), suggesting that activated Tcf can affect the position of this hindbrain marker rather at the onset than at the end of gastrulation. In contrast, *dnXWnt8* mRNA resulted in a robust posteriorward expansion of *Otx2* and a posterior shift of *En2* and *Krox20* expression (Fig. 1K–K″), confirming a role of Wnt signals in posterior neural development. In most of the experiments, the anterior borders of the telencephalon marker *FoxG1* and the spinal cord marker *HoxC6* were not affected (Fig. 1H′–K′,H″–K″; see Fig. S1C,C',D,D' in the Supplementary material), suggesting that the effects of altering Hh and Wnt signaling were confined to the forebrain, midbrain and hindbrain territories of the neural plate. To investigate whether the effects of Hh and Wnt signals on neural plate patterning correlate with changes in the mesoderm, we performed double-*in situ* hybridization with *Otx2* and the paraxial mesoderm marker *MyoD*. We found that animal injection of *XGli1* mRNA, *Gli3C′ΔClaI* mRNA, *XWnt3a* DNA, and *dnXWnt8* mRNA affected the anteroposterior extension of the paraxial mesoderm to an extent that was less pronounced than for the neural markers (see Fig. S3 in

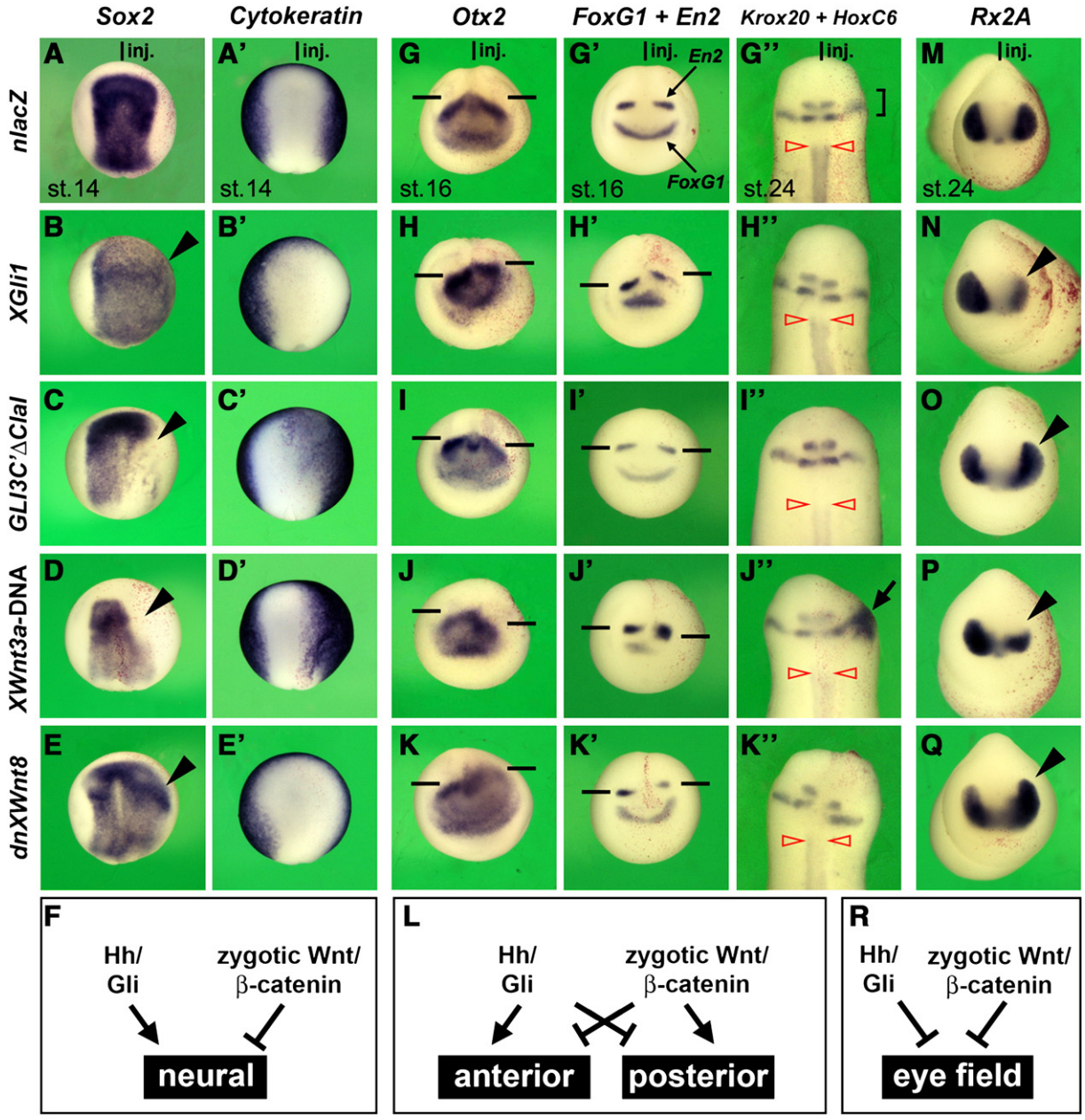


Fig. 1. Function of Hh and Wnt signals during neural induction, anteroposterior patterning and eye field development. *Xenopus* embryos were injected into the animal pole of a single blastomere at the 4-cell stage with the indicated constructs and *nlacZ* mRNA as a lineage tracer (red nuclei on injected right side). (A,A′) Control late gastrula in dorsal view depicting the neural plate marker *Sox2* and the epidermal marker *Cytokeratin*. (B,B′,C,C′) *XGli1* mRNA expands neural at the expense of epidermal tissue, while *Gli3C′ΔClaI* mRNA causes the opposite effect. (D,D′,E,E′) *XWnt3a* DNA promotes epidermal and *dnXWnt8* mRNA neural development. (G–G″) Control embryos at neurula stage in anterior view (G,G′) and at tail bud stage in dorsal view (G″). *Otx2* demarcates the developing cement gland, forebrain and midbrain (horizontal line), *FoxG1* the telencephalon, *En2* the posterior midbrain, and *Krox20* the hindbrain rhombomeres 3 and 5 (bracket). Open arrowhead points to the anterior border of *HoxC6* expression in the spinal cord. (H–H″,I–I″) *XGli1* mRNA anteriorizes the brain, whereas *Gli3C′ΔClaI* causes very subtle posteriorization. (J–J″) *XWnt3a* DNA has posteriorizing activity. The arrow points to an expansion of *Krox20* expression in neural crest cells. (K–K″) *dnXWnt8* mRNA leads to anteriorization. (M) Control tail bud embryo in anterior view, showing *Rx2A* expression in the bilateral eyes. (N–O) *XGli1* mRNAs diminish, whereas *Gli3C′ΔClaI* mRNAs expand the eye anlage. (P,Q) *XWnt3a* DNA leads to a smaller and *dnXWnt8* mRNA to a larger eye anlage. (F,L,R) Summary of effects of Hh and zygotic Wnt signals on neural induction (F), anteroposterior patterning (L), and specification of the eye field (R). The indicated gene expression patterns were obtained in: B, 43/46; B′, 24/26; C, 48/49; C′, 41/52; D, 36/40; D′, 17/19; E, 57/57; E′, 64/68, H, 9/10; H′, 12/12 (*FoxG1*), 8/12 (*En2*); H″, 7/7 (*Krox20*), 11/11 (*HoxC6*); I, 11/13; I′, 18/20 (*FoxG1*), 15/20 (*En2*); I″, 17/26 (*Krox20*), 45/49 (*HoxC6*); J, 44/44; J′, 34/34 (*FoxG1*), 29/34 (*En2*); J″, 43/50 (*Krox20*), 12/17 (*HoxC6*); K, 42/45; K′, 37/37 (*FoxG1*), 29/37 (*En2*); K″, 15/15 (*Krox20*), 13/13 (*HoxC6*); N, 8/10; O, 17/20; P, 8/9; Q, 11/11 (*Rx2A*).

the Supplementary material), suggesting that cell fate changes rather than morphogenetic defects account for the observed effects on neural plate patterning. Hence Hh/Gli are potent anteriorizing signals, whereas Wnt/β-catenin signals exert important roles in suppressing anterior and stimulating posterior neural development (Fig. 1L).

The bilateral eyes are ectodermally-derived organs that originate from the anterior neural plate. Overexpression of a constitutively activated Gli protein or Sonic Hedgehog can impair with early eye development (Cornesse et al., 2005; Marine et al., 1997), but it is not well understood whether Hh/Gli signals have a function in the specification of the eye field. In addition, little is known about temporal aspects of canonical Wnt signals in eye field specification. We found that *XGli1* or *XBhh* mRNAs decreased the eye size on the injected side, while *Gli3C′ΔClaI* and *XPtc1ΔLoop2* mRNAs enlarged the *Rx2A*-positive eye anlagen (Fig. 1N,O; see Fig. S1E,F in the Supplementary material). *XWnt3a* DNA reduced *Rx2A* expression particularly in the posterior

domain (Fig. 1P). THVGR also downregulated this marker when activated at stage 10 or at stage 14 (see Fig. S2D′,E′ in the Supplementary material), suggesting that Wnt/Tcf signals can suppress the formation of the eye field in gastrula and early neurula embryos. It is noteworthy that the activation of Wnt signaling at stage 14 caused a robust anteriorward shift of *Rx2A* expression, while the posteriorizing effects on the hindbrain marker *Krox20* had only been moderate (Fig. S2C′,E′ in the Supplementary material), supporting the idea that the eye and hindbrain fields may be independently regulated. In contrast, *dnXWnt8* mRNA caused an expansion of *Rx2A* expression (Fig. 1Q). These observations allow the conclusion that both Hh/Gli and Wnt/β-catenin signals negatively regulate the eye field (Fig. 1R).

Cloning and expression of Xenopus Sufu

Because of the well-established role of Suppressor-of-fused (Sufu) in the regulation of Hh/Gli signaling in the mouse (Cooper et al., 2005; Svärd et al., 2006; Varjosalo et al., 2006) and a previous *in vitro* report that links Sufu to Wnt/β-catenin signaling (Meng et al., 2001), we cloned the homolog of Sufu in *Xenopus laevis* (*XSufu*; see Fig. S4 in the Supplementary material and Supplemental experimental procedures). RT-PCR revealed abundant *XSufu* transcripts from the 4-cell to tadpole stage, indicating maternal and zygotic expression (Fig. 2A). Equal *XSufu* mRNA levels were seen in animal and vegetal portions of blastula embryos (Fig. 2B), and comparable amounts were detected in dorsal and ventral marginal zone explants of early gastrula embryos (Fig. 2C). *In situ* hybridization showed robust *XSufu* expression in advanced gastrula embryos in a crescent-shaped anterior ectoderm domain comprising the panplacodal primordium (Fig. 2D,E). Moderate expression levels were also found in the neural plate with exclusion of the midline. During neurulation, distinct *XSufu* signals were observed in the anterior neural plate, neural crest, and in a bilateral row of cells adjacent to the neural groove (Fig. 2F–I). After closure of the neural tube, *XSufu* is expressed in the anterior brain, eye field, and migrating neural crest cells (Fig. 2J). In tailbud embryos, transcripts were localized in the forebrain, midbrain–hindbrain boundary, eye and ear vesicles, branchial arches, and tailbud (Fig. 2K–M). Together, these data show that *XSufu* is ubiquitously expressed in embryos up to the early gastrula stage and later restricted to distinct ectodermal derivatives.

Overexpression of XSufu suppresses neural fate, affects anteroposterior patterning, and promotes eye specification

To investigate the activity of XSufu during *Xenopus* development, we microinjected *XSufu* mRNA into the animal pole of a single dorsal blastomere at the 4-cell stage (Fig. 3). In early neurula embryos, *XSufu* mRNA led to a significant reduction of *Sox2* expression in the neural plate concomitant with an expansion of the epidermis marker *Cytokeratin* (Fig. 3A′,B′). *XSufu* mRNA caused a posteriorward displacement of *Otx2* (forebrain and midbrain anlage), *FoxG1* (telencephalon), *En2* (posterior midbrain), and *Krox20* expression (hindbrain) (Fig. 3C′–E′). A clear dose-dependent effect could be observed, with 100 pg *XSufu* mRNA causing only a little posteriorward shift, and 1600 pg *XSufu* mRNA resulting in shifts of up to two rhombomeric units (see Fig. S5 in the Supplementary material). Co-injection of *XSufu* mRNA and *XWnt3a* DNA restored normal expression of *Otx2*, *FoxG1*, *En2*, and *Krox20* (Fig. 3C″,D″,E″), suggesting that the anteriorization observed upon XSufu overexpression may be mediated through inhibition of Wnt signaling. Animal injection of *XSufu* mRNA did not alter mesodermal *MyoD* expression (Fig. 3F′), suggesting that overexpression of XSufu may directly affect ectodermal patterning. In tailbud embryos, *XSufu* mRNA caused an enlargement of the eye-specific *Rx2A* expression domain (Fig. 3G′). We also observed an enlargement of pigmented eye vesicles and thickening of the neural retina at the tadpole stage (Fig. 3H,I,I′,J,J′), supporting a positive effect of XSufu on eye development.

Knockdown experiments support roles of XSufu in regulation of neural fate, anteroposterior patterning and eye development

To study the endogenous function of XSufu, we designed a specific 25-mer morpholino oligonucleotide sequence directed against the translation initiation site of the *XSufu* gene (*XSufu*-MO, Fig. 4A). In an *in vitro* transcription–translation assay, the *XSufu*-MO effectively blocked protein synthesis of XSufu, whereas a non-specific control-MO had no effect (Fig. 4B, lanes 1–3). The specificity of the *XSufu*-MO was corroborated by its inability to suppress translation of a recombinant *XSufu** construct, which lacks the 5′UTR target sequence and therefore is not targeted by the *XSufu*-MO (Fig. 4A,B, lanes 4 and 5). Microinjection of *XSufu*-MO into the animal pole of 2-cell stage embryos led to microcephaly and shortened tail structures

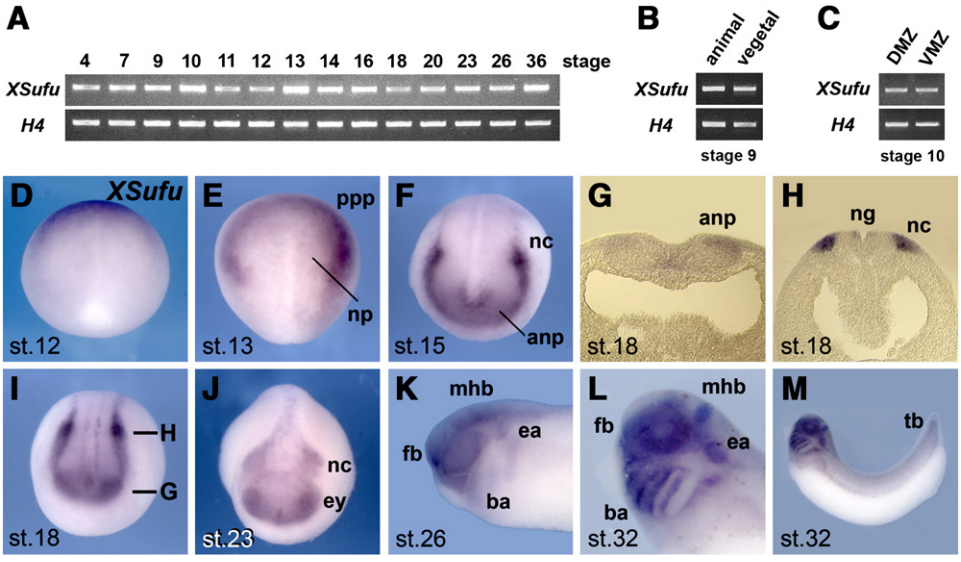


Fig. 2. Gene expression of *XSufu* in *Xenopus* embryos. (A–C) RT-PCR of whole embryos (A) and embryonic explants (B,C). *Histone H4* was used as RNA loading control. DMZ, dorsal marginal zone; VMZ, ventral marginal zone. (D–M) Whole-mount *in situ* hybridization of embryos shown in dorsal (D,E), anterior (F,I,J), and lateral views (K–M). Panels (G) and (H) are transversal sections of embryo in (I). anp, anterior neural plate; ba, branchial arch; ea, ear; ey, eye; fb, forebrain; mhb, mid–hindbrain boundary; nc, neural crest; ng, neural groove; np, neural plate; ppp, panplacodal primordium, tb, tail bud.

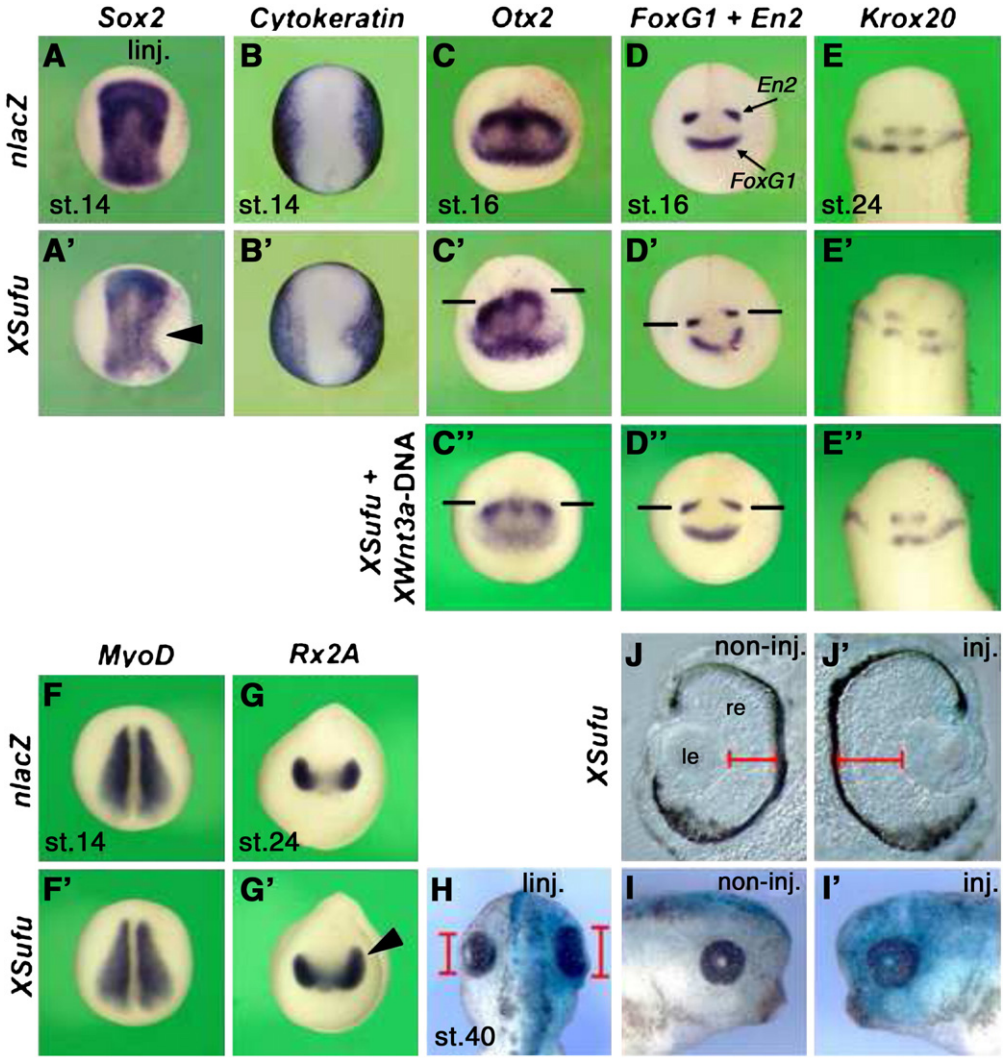
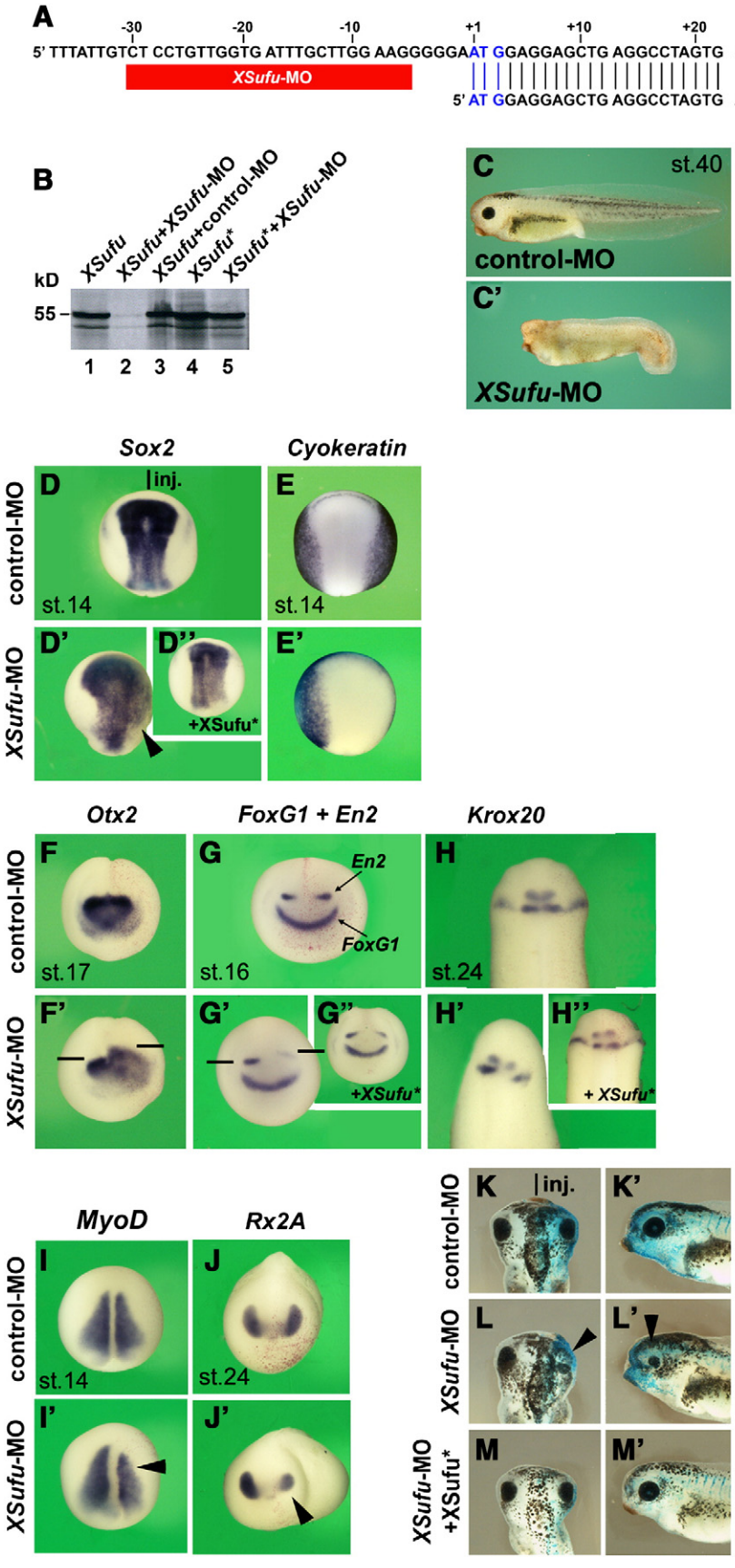


Fig. 3. Microinjection of *XSufu* mRNA suppresses neural plate formation, stimulates anterior neural markers and promotes eye development. Embryos were animally injected at the 4-cell stage with *nlacZ* mRNA as control or *XSufu* mRNA. All specimens were injected into a single blastomere. Embryos are shown in dorsal (A,A′,B,B′,E–E″,F,F′,H), anterior (C–C″,D–D″,G,G′), lateral views (I,I′), or as transversal section (J,J′). (A,B,A′,B′) *XSufu* mRNA causes reduction of *Sox2* and concomitant expansion of *Cytokeratin* expression on the injected right side (arrowhead). (C–E,C′–E′) *XSufu* mRNA causes posteriorward expansion of *Otx2* expression and a posterior shift of *En2* and *Krox20* expression, while *FoxG1* expression remains unaffected. (C″–E″) Co-injection of *XSufu* mRNA and *Wnt3a* DNA restores normal expression of *Otx2*, *En2*, and *Krox20*. (F,F′) *XSufu* mRNA has no effect on *MyoD* expression upon animal injection. (G,G′,H–J,I′,J′) *XSufu* mRNA leads to an enlargement of *Rx2A* expression, enlarged eye structures and expansion of the neural retina. Frequency of embryos with the indicated phenotype was: A′, 44/46; B′, 61/62; C′, 7/10; C″, 12/12; D′, 46/51; D″, 19/21; E′, 116/125; E″, 32/40; F′, 19/21; G′, 95/108; H, 56/115.

(Fig. 4C′). XSufu-morphant tadpoles did not swim and failed to show avoidance reflexes (data not shown). A single injection of *XSufu*-MO caused a significant expansion of *Sox2* at the expense of *Cytokeratin* expression (Fig. 4D′,E′). These effects are opposite to those obtained with *XSufu* mRNA (Fig. 3A′,B′) and suggest an important function of XSufu in restricting neural plate development. Depletion of *XSufu* resulted in an expansion of *Otx2* expression concomitant with a reduction of *En2* and posterior shift of *En2* and *Krox20* expression in the developing brain (Fig. 4F′–H′). These effects are similar to those observed in gain-of-function studies (Fig. 3C′–E′), suggesting that XSufu may regulate anteroposterior patterning of the neural plate in a biphasic manner (see Discussion). Of note, knockdown of *XSufu* led to a reduction and posteriorward retraction of the mesodermal marker *MyoD* (Fig. 4I′), raising the question of whether the change in the position of neural markers may be linked to indirect effects through changes in the mesoderm? We therefore injected *XSufu*-MO

Fig. 4. Depletion of XSufu induces expansion of the neural plate, stimulates anterior neural markers and suppresses eye development. (A) Targeting sequence of the *XSufu* morpholino oligonucleotide (*XSufu*-MO). The non-targeted *XSufu** mRNA construct lacks the 5′ untranslated region. Blue letters indicate start codon. (B) *In vitro* transcription–translation assay. The *XSufu*-MO, but not an unspecific control-MO, inhibits XSufu protein synthesis. *XSufu*-MO does not reduce translation of *XSufu** mRNA. (C,C′) At the tadpole stage, *XSufu*-MO-injected embryos have reduced head and tail structures. (D–D″) Dorsal view of early neurula embryos. Injection was performed animally into one blastomere at the 2-cell stage together with *nlacZ* mRNA as tracer. *XSufu*-MO, but not control-MO, causes expansion of *Sox2* expression on the injected side (arrowhead); this effect is reverted by co-injection with *XSufu** mRNA. (E,E′) *XSufu*-MO leads to a reduction of *Cytokeratin* expression. (F–H,F′–H′,G″,H″) Anterior view of neurula (F,F′,G–G″) and dorsal view of tailbud embryo (H–H″). *XSufu*-MO causes expansion of *Otx2*, reduced intensity of *En2*, and a posterior shift of *En2* and *Krox20* expression, while *FoxG1* expression remains unaffected. Co-injection of *XSufu*-MO and *XSufu** mRNA rescues normal *En2* and *Krox20* expression. (I,I′) Dorsal view of early neurula. *XSufu*-MO induces reduction of *MyoD* expression (arrowhead). (J,J′) Anterior view of tail bud embryo. *XSufu*-MO leads to a reduction of *Rx2A* expression. (K–M,K′–M′) A single injection of *XSufu*-MO causes reduction of eye structures (arrowheads), which is reverted by co-injection of *XSufu** mRNA. The indicated phenotypes were observed in: C, 70/83; C′, 21/43; J, 22/24; D, 50/50; D′, 71/77; D″, 82/87; E, 24/27; E′, 39/40; F, 23/24; F′, 20/20; G, 39/39 (*FoxG1*), 55/55 (*En2*); G′, 42/42 (*FoxG1*), 54/54 (*En2*); G″, 31/31; H, 65/67; H′, 79/79; H″, 34/40; I, 24/26; I′, 27/28; J, 40/42; J′, 67/73; L,L′, 25/25; M,M′, 21/43.

into neural-fated A1 blastomeres at the 32-cell stage to avoid an effect on mesoderm. A posteriorward expansion of *Otx2* was observed, while *MyoD* expression remained unaffected (see Fig. S6 in the Supplementary material), suggesting that XSufu may exert its anteriorizing function directly in the neural plate. Furthermore, *XSufu*-MO caused a reduction of the eye-specific *Rx2A*



expression domain at the early tailbud stage (Fig. 4J′) and microphthalmy in tadpole embryos (Fig. 4L,L′). These results contrast the enlarged eye structures obtained in overexpression experiments (Fig. 3G′,H,I,I′,J,J′), supporting a positive function of XSufu during eye development. Microinjection of control-MO showed no phenotype (Fig. 4C–K,K′), and co-injection of *XSufu** mRNA with *XSufu*-MO restored normal expression of selected marker genes (*Sox2*, *FoxG1*, *En2*, and *Krox20*) and eye structures (Fig. 4D″,G″,H″,M,M′), verifying the specificity of the loss-of-function experiments.

We next addressed the impact of Hh and Wnt signaling on mediating the effects of XSufu depletion. Inhibition of Hh signals by *GLI3C′ΔClaI* mRNA in *XSufu*-morphant embryos restored normal expression of *Sox2*, *Otx2*, *FoxG1*, *En2*, and *Krox20* (see Fig. S7A–D in the Supplementary material), suggesting a role of XSufu as negative regulator of Hh signaling during neural induction and anteroposterior patterning. On the other hand, co-injection of *XSufu*-MO with either *GLI3C′ΔClaI* or *dnXWnt8* mRNA restored normal *Rx2A* expression (see Fig. S7E,F in the Supplementary material), implying that the function of XSufu in the specification of the eye field may be mediated through inhibition of both Hh and Wnt signals. Together, XSufu has critical functions in suppressing neural fate, ensuring proper anteroposterior patterning, and promoting eye specification.

XSufu regulates cement gland, neural crest, neuron, and muscle development

Formation of the cement gland is inhibited by Wnt signals (Christian and Moon, 1993), but dependent on Hh signals (Lai et al., 1995). A single animal injection of *XSufu* mRNA resulted in reduction of the cement gland marker *XAG*, an effect that was also obtained upon injection of *XSufu*-MO (Fig. 5A′,B′), suggesting that proper levels of XSufu protein are important for cement gland formation.

Induction of neural crest cells is negatively regulated by Hh/Gli signals (Brewster et al., 1998; Franco et al., 1999; Marine et al., 1997), but dependent on Wnt signals (Deardorff et al., 2001; LaBonne and Bronner-Fraser, 1998; Wu et al., 2005). Both upregulation and downregulation of XSufu caused reduction of the neural crest markers *Slug* and *Snail* (Fig. 5C′,D′,E′,F′). The rescue of *Slug* and *Snail* expression by co-injected *XSufu** mRNA underscored the specificity of the *XSufu*-MO effect (Fig. 5D″,F″). To investigate of whether effects on neural crest markers are a secondary consequence of changes in the mesoderm, we microinjected *XSufu*-MO at the 32-cell stage into animal A2 blastomeres fated to become neural crest cells. We observed reduction of *Slug* and *Snail* expression (see Fig. S6B′,C′ in the Supplementary material) suggesting that XSufu may directly promote neural crest fate. In *XSufu*-morphant embryos, co-injected *GLI3C′ΔClaI* mRNA restored normal *Slug* and *Snail* expression (see Fig. S7G,H in the Supplementary material), suggesting a role of XSufu as inhibitor of Hh signaling during neural crest development.

Activation of Hh/Gli signaling has a negative impact on primary neurogenesis and myogenesis (Franco et al., 1999; Marine et al., 1997). In contrast, Wnt/β-catenin signals are required for sensory neuron (Garcia-Morales et al., 2009; Marcus et al., 1998) and muscle development (Hoppler et al., 1996; Leyns et al., 1997; Salic et al., 1997). Animal injection of *XSufu* mRNA slightly expanded *N-tubulin* expression (Fig. 5G′), while knockdown of *XSufu* led to loss of *N-tubulin*-positive neurons (Fig. 5H′). We also observed reduction of the muscle-specific marker *MyoD* and impaired somite formation at the tailbud stage (Fig. 5I′), indicating that the paralysis observed in *XSufu* morphant larvae (Fig. 4C′) might be caused by a failure of proper innervation and/or muscle formation. The results suggest that XSufu exerts a critical role in promoting primary neuron and muscle development likely through inhibiting Hh/Gli signaling.

We note that *XSufu*-MO and *XSufu* mRNA had no detectable effect on proliferation and apoptosis in neurula and tailbud embryos, as shown by immunohistochemical pH3 staining and the TUNEL assay (see Figs. S8 and S9 in the Supplementary material). In sum, XSufu is essential for the specification of cement gland, neural crest, neuronal and muscle cell fate.

XSufu blocks Hh/Gli and Wnt/β-catenin signaling

We next analyzed the interaction of XSufu with Hh and Wnt signaling (Figs. 6, 7). RT-PCR analysis showed that *XSufu* lowered *XBhh*-induced expression of *Xenopus Patched 1* (*XPtc1*), *XPtc2*, and *XGli1* in mRNA-injected animal caps (Fig. 6A, compare lanes 3 and 4). As the *XSufu* gene is expressed in the animal hemisphere at blastula stage (Fig. 2B), we investigated of whether endogenous XSufu is needed to suppress Hh/Gli signaling. *XSufu*-MO, but not the unspecific control-MO, elevated transcript levels of the Hh target genes in animal cap explants (Fig. 6A, compare lanes 5 and 6). Whole-mount *in situ* hybridization of neurula stage embryos further revealed that animally injected *XSufu* mRNA reduced and *XSufu*-MO expanded *XPtc1* expression in anterior and paraxial domains of the neural plate (Fig. 6B,B′,C,C′), suggesting that XSufu is not only a potent, but necessary inhibitor of Hh signaling.

RT-PCR analysis of animal cap explants at the gastrula stage revealed that *XSufu* mRNA inhibited *XWnt8*-induced expression of *Siamois* and *Xenopus nodal-related 3* (*Xnr3*) in a dose-dependent manner (Fig. 7A, compare lanes 3–5). In contrast, *XSufu*-MO, but not control-MO, caused robust induction of *Siamois*, *Xnr3* and *Chordin* expression (Fig. 7B, compare lanes 3 and 4). In whole-mount embryos, marginal injection of *XSufu* mRNA reduced *Xnr3* and *Chordin* transcripts in the dorsal blastopore lip, whereas animally injected *XSufu*-MO led to an expansion of these organizer markers (Fig. 7C–F,C′–F′). *XSufu* mRNA also reduced ectopic expression of *Xnr3* and counteracted dorso-anteriorizing development induced by animal injection of *XWnt8* mRNA (Fig. 7G–G″; see Fig. S10 in the Supplementary material).

A recent study proposed that Gli proteins may promote Wnt signaling in *Xenopus* embryos through a mechanism in which XGli2 and XGli3 induce transcription of *XWnt8* and other Wnt genes (Mullor et al., 2001). To investigate whether the inhibitory effect of XSufu on Wnt signaling is direct or mediated through modulation of Gli proteins, we studied the interaction of XWnt8, XSufu and XGli in *Xenopus* embryos (Fig. 7H–M). Co-injection of *XWnt8* and *XSufu* mRNAs into a single ventral blastomere at the 4-cell stage reverted the formation of a secondary body axis that is induced by *XWnt8* mRNA injection alone (Fig. 7H–J,N, lanes 1–3). A triple injection of *XWnt8*, *XSufu* and *XGli1* mRNAs did not restore but further suppressed second axis induction (Fig. 7K,N, lanes 4,5), suggesting that XSufu may not antagonize Wnt signaling through inhibition of XGli1. Interestingly, *XGli1* mRNA alone also impaired *XWnt8*-induced axis duplication, as indicated by the shortening or absence of second axes upon co-injection of *XWnt8* with increasing amounts of *XGli1* mRNA (Fig. 7L,M, N, lanes 6,7). We had previously shown that *XSufu* mRNA caused a posteriorward shift of the brain markers *Otx2*, *En2*, and *Krox20* in neurula and tailbud embryos (Fig. 3C′–E′), and that co-injection of *XSufu* mRNA and *XWnt3a* DNA reverted the anterior shift of these marker genes (Fig. 3C″–E″) that was induced by *XWnt3a* DNA alone (Fig. 1J–J″). We now found that overexpression of *XGli1* mRNA did not rescue the inhibition of *XSufu* mRNA on *XWnt3a* DNA-induced posteriorization of the neural plate (see also Fig. S11A–C in the Supplementary material). Moreover, *XGli1* mRNA caused a posterior shift of *Otx2*, *En2*, and *Krox20* expression not only when injected alone (Fig. 1H–H″) but also when *XGli1* mRNA was co-injected with *XWnt3a* DNA (see also Fig. S11A′–C′ in the Supplementary material). We conclude that XSufu may directly regulate Wnt signaling and that XGli1 can inhibit Wnt responses during secondary axis induction and neural patterning. Together, our data suggest that XSufu is a dual regulator of Hh/Gli1 and Wnt/β-catenin signaling in the *Xenopus* embryo.

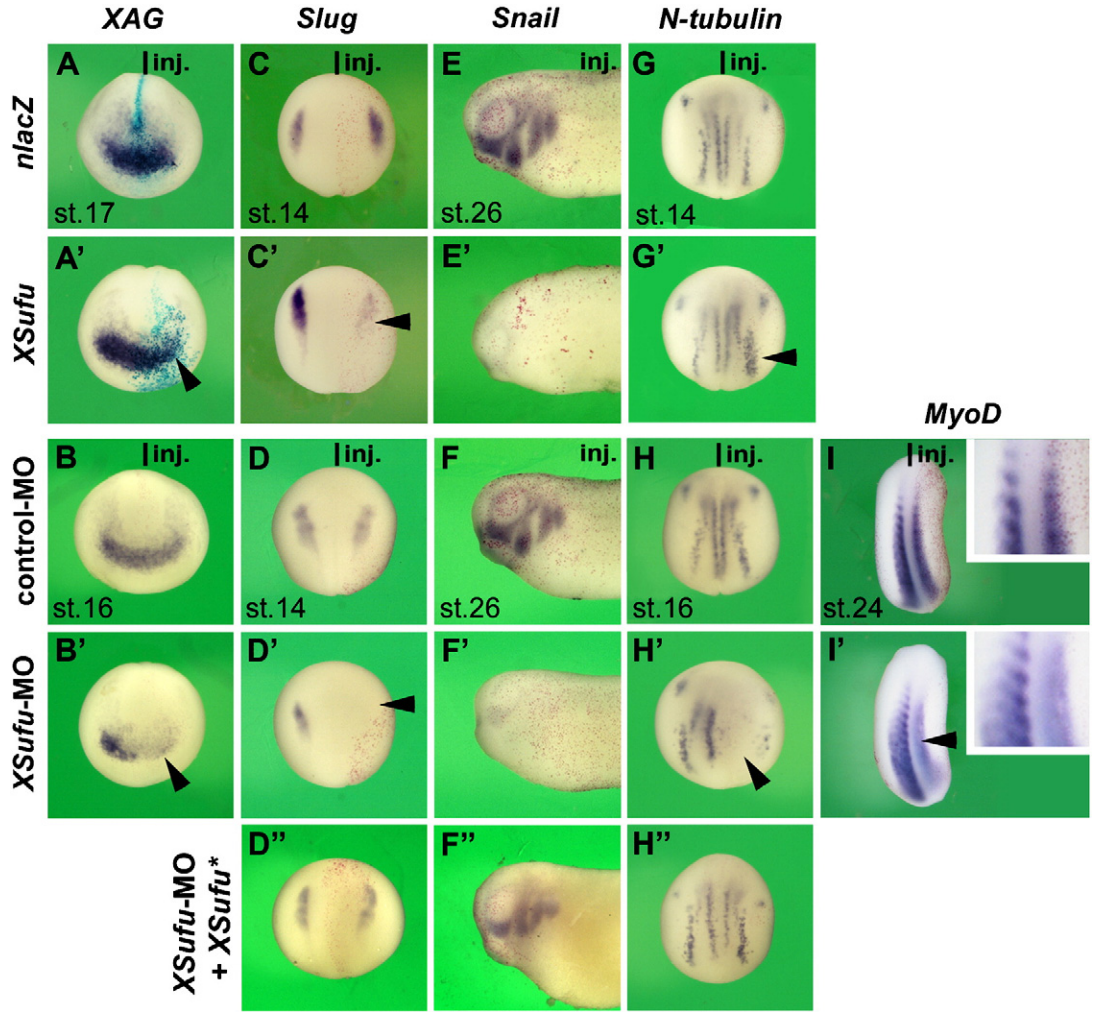


Fig. 5. *XSufu* is important for cement gland, neural crest, neuronal and paraxial mesoderm development. Embryos were injected together with *nlacZ* mRNA as tracer animally into one blastomere at the 2- or 4-cell stage. (A,A′,B,B′) Whole-mount *in situ* hybridization of early neurula embryos in anterior view. *XSufu* mRNA and *XSufu*-MO lead to a reduction of the cement gland marker *XAG* while a non-specific control-MO has no effect. (C–F,C′–F′,D″,F″) Late gastrulae in dorsal and tail bud embryo in lateral view. *XSufu* mRNA and *XSufu*-MO cause a reduction of the neural crest markers *Slug* and *Snail*. Co-injection of *XSufu*-MO and *XSufu** mRNA reverts to normal *Slug* and *Snail* expression. (G,G′,H–H″) Early neurulae in dorsal view. *XSufu* mRNA causes slight expansion of the neuronal marker *N-tubulin*. *XSufu*-MO reduces *N-tubulin* expression. Normal *N-tubulin* expression is seen after co-injection of *XSufu*-MO with *XSufu** mRNA. (I–I′) Tail bud embryos in dorsal view. *XSufu*-MO inhibits formation of *MyoD*-positive segmented somites (see also magnification in inset). Indicated effects were observed in: A′, 9/16; B, 40/45; B′, 7/7; C′, 70/70; D, 65/68; D′, 14/15; D″, 62/64; E′, 62/65; F, 13/13; F′, 19/19; F″, 30/33; G′, 66/81; H, 102/105; H′, 78/84; H″, 31/39; I, 15/20; I′, 17/17.

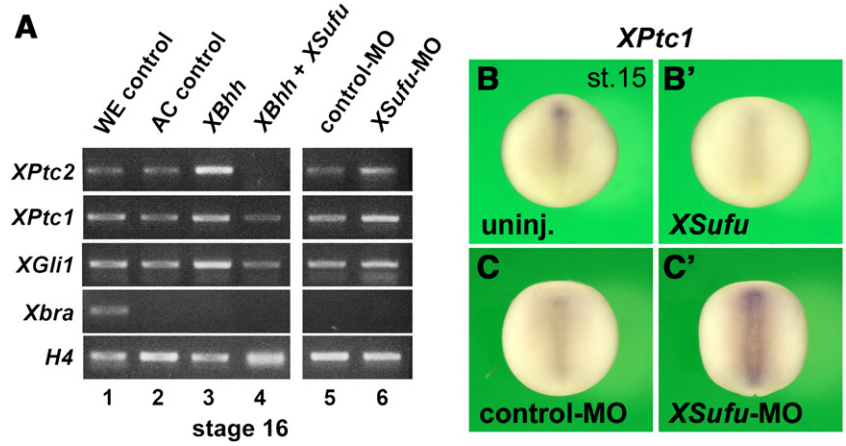


Fig. 6. XSufu inhibits Hh/Gli signaling in the *Xenopus* embryo. (A) Molecular analysis by RT-PCR of animal cap (AC) explants cultured until stage 16. Animal injection of 500 pg *XBhh* mRNA elevates the expression of *XPtc2*, *XPtc1*, and *XGli1*, and this effect is reverted by co-injection of 16 ng *XSufu* mRNA. *XSufu*-MO, but not control-MO, increases the expression of these Hh target genes. *H4* was used for normalization. (B,B′,C,C′) Whole-mount *in situ* hybridization of early neurula embryos in dorsal view. Animal injection of 15 ng *XSufu* mRNA reduces, while *XSufu*-MO, but not control-MO, expands the expression of *XPtc1* expression. Indicated effects were observed in: B, 61/61; B′, 29/38; C, 58/58; C′, 32/37.

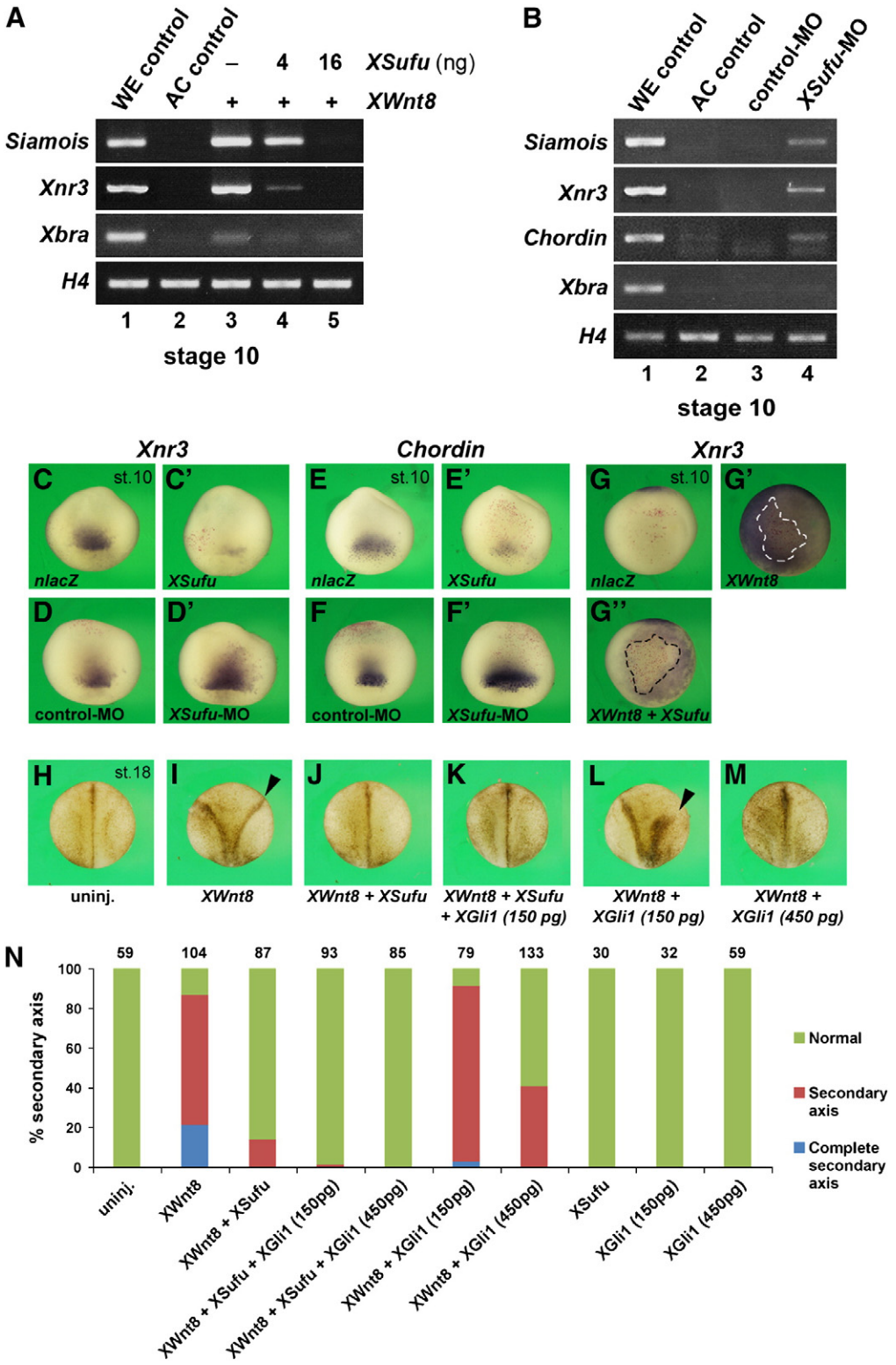


Fig. 7. XSufu inhibits Wnt/β-catenin signaling in the *Xenopus* embryo. (A) Molecular analysis by RT-PCR of animal cap (AC) explants cultured until stage 10. Embryos were animally injected with 25 pg *XWnt8* mRNA either alone or in combination with the indicated amount of *XSufu* mRNA. *H4* was used for normalization. *XSufu* mRNA downregulates *XWnt8*-induced transcription of *Siamois* and *Xnr3* in a dose-dependent manner. (B) *XSufu*-MO, but not control-MO, induced expression of the Wnt target genes *Siamois*, *Xnr3*, and *Chordin* in injected AC explants at stage 10. (C–F,C′–F′) Whole-mount *in situ* hybridization of early gastrula embryos in dorsal view. A single marginal injection of *XSufu* mRNA at the 4-cell stage reduces, while a single animal injection of *XSufu*-MO, but not of control-MO, at the 2-cell stage expands endogenous expression of *Xnr3* and *Chordin*. (G–G″) Animal view of early gastrulae. A single animal injection of 12 pg *XWnt8* mRNA at the 4-cell stage induces ectopic *Xnr3* expression (G′) that is reduced by co-injection of *XWnt8* and *XSufu* mRNA (G″). Injected *nlacZ*-positive cells are highlighted with a strippled line. (H) Dorsal view of uninjected neurula embryo. Condensed natural pigmentation at the site of neural tube closure delineates the main body axis. (I–M) A single ventral injection of 4 pg *XWnt8* mRNA induces a complete secondary body axis (arrowhead in I) that is blocked by co-injection with 4 ng *XSufu* mRNA (J) or by co-injection with a combination of *XSufu* and 150 pg *XGli1* mRNA (K). Note that *XWnt8*-induced secondary axis formation is partly reversed by co-injection of 150 pg *XGli1* mRNA (arrowhead in L) and inhibited by co-injection of 450 pg *XGli1* mRNA (M). (N) Quantification of secondary axis formation. Indicated effects were observed in: C, 14/14; C′, 8/10; D, 15/18; D′, 24/36; E, 13/13; E′, 7/8; F, 24/26; F′, 21/28; G, 47/47; G′, 14/14; G″, 55/55.

Physical interaction of XSufu with XGli1 and Xβ-catenin proteins

In view of these lines of embryological evidence linking XSufu to Hh and Wnt signaling, we addressed the biochemical interaction of XSufu with intracellular components of the pathways that transduce the signals to the nucleus. XGli1 is the main transactivator of Hh signaling in *Xenopus* (Lee et al., 1997), and mammalian Sufu has been shown to physically associate with Gli1 (Dunaeva et al., 2003; Kogerman et al., 1999; Merchant et al., 2004). In response to the activation of the Wnt signaling pathway, stabilized β-catenin regulates the transcription of Wnt target genes, and mammalian Sufu inhibits β-catenin activity through complex formation (Meng et al., 2001). In HEK293 cells, we expressed flag-tagged XSufu (XSufu-flag), myc-tagged XGli1 (XGli1-myc), and myc-tagged Xβ-catenin that is stabilized by four point mutations in aminoterminal GSK-3 phosphorylation sites (Xptβ-catenin-myc; Yost et al., 1996). Careful titration of plasmid DNA amounts and adjustment of culture conditions allowed us to obtain equivalent protein levels of XGli1-myc and Xptβ-catenin-myc as judged by Western blot analysis with anti-myc antibodies (Fig. 8A, middle panel, compare lanes 3 and 5). Using co-immunoprecipitation, we found that XSufu-flag immunoprecipitated with comparable levels of XGli1-myc and Xptβ-catenin-myc (Fig. 8A, upper panel). Hence XSufu can bind to XGli1 and Xptβ-catenin proteins.

Sufu is essential for the crosstalk between Hh/Gli and Wnt/β-catenin signaling

We investigated the interaction of Hh/Gli and Wnt/β-catenin signaling and asked of whether Sufu plays a role in regulating the crosstalk between the two pathways. To this end we microinjected *XGli1* and Xpt*β-catenin* mRNAs with specific luciferase reporter constructs into the animal pole of *Xenopus* embryos at the 4-cell stage that were independently injected with control-MO or *XSufu*-MO at the 2-cell stage. At the early gastrula stage, *XGli1*-induced activation of the Hh reporter construct *8x3′Gli-BS luc* (Sasaki et al., 1997) was further increased 2-fold by overexpressing *XGli1* with *Xptβ-catenin* in control-MO-injected embryos (Fig. 8B). However, *Xptβ-catenin* failed to increase *XGli1*-induced Hh pathway activation in *XSufu*-morphant embryos (Fig. 8C). On the other hand, *Xptβ-catenin*-induced activation of the Wnt reporter TOP-flash (Upstate Biotechnology) was reduced to one third by overexpressing *XGli1* with *Xptβ-catenin* in control-MO-injected embryos (Fig. 8D). Of note, *XGli1* did not significantly reduce *Xptβ-catenin*-induced Wnt pathway activation in *XSufu*-morphants (Fig. 8E). We did not observe activation of the *8x3′mGli-BS luc* reporter in which the Gli binding sites are mutated (Sasaki et al., 1997) nor activation of *FOP-flash* (Upstate Biotechnology), an altered *TOP-flash* construct in which the Tcf binding sites are inactivated by point mutations (Fig. 8B–E). Next we transfected mouse *Gli1* and stabilized mouse *ΔN89β-catenin* (Meng et al., 2001) with the luciferase reporter constructs into MEF cells that were wild type (MEF WT) or homozygous mutant for the *Sufu* gene (MEF *Sufu*−/−; Svärd et al., 2006). We found that *ΔN89β-catenin* increased *Gli1*-induced *8x3′Gli-BS luc* activity in MEF WT (Fig. 8F), but not in MEF *Sufu*−/− cells (Fig. 8G). Co-transfection of MEF *Sufu*−/− cells with a moderate amount of mouse *Sufu* plasmid restored the stimulating effect of *ΔN89β-catenin* on *Gli1*-induced Hh reporter activity (Fig. 8H). In turn, *Gli1* suppressed *ΔN89β-catenin*-induced *TOP-flash* activity in MEF WT (Fig. 8I), but not significantly in MEF *Sufu*−/− cells (Fig. 8J). Addition of *Sufu* to MEF *Sufu*−/− rescued the inhibitory effect of *Gli1* on *ΔN89β-catenin*-induced Wnt reporter activity (Fig. 8K). Similarly, *Xptβ-catenin* stimulated Hh pathway activation by *XGli1* and, vice versa, *XGli1* inhibited Wnt pathway activation by *Xptβ-catenin* in transfected NIH3T3 cells (Fig. S12A,B in the Supplementary material). Western blot analysis verified the presence of endogenous Sufu protein in NIH3T3 and MEF WT, and confirmed its absence in MEF *Sufu*−/− cells (see Fig. S12C in the Supplementary material). In sum, our data suggest a conserved crosstalk in which exogenous Wnt/β-catenin stimulates Hh/Gli signaling under overexpression of Gli1, and in turn exogenous Hh/Gli inhibits Wnt/β-catenin signaling under overexpression of stabilized β-catenin. In both *Xenopus* embryos and mammalian cells, Sufu is critically involved in the interaction between Hh/Gli and Wnt/β-catenin signaling (Fig. 8L).

Discussion

In this study, we investigated the role of *Xenopus* Sufu (XSufu) in the early embryo. Our gain- and loss-of-function studies revealed essential functions of XSufu in diverse aspects of ectodermal patterning, including the restriction of the neural plate, positioning of brain compartments along the anteroposterior body axis, and stimulation of eye development. Additional functions of XSufu could be described for cement gland, neural crest, neuronal, and muscle development. We provided evidence that XSufu acts as an inhibitor of Hh and Wnt signaling in the *Xenopus* embryo. Our data further show that Gli1 suppresses Wnt signaling under overexpression of β-catenin, and β-catenin in turn stimulates Hh signaling under overexpression of Gli1. Importantly, the presence of endogenous Sufu is vital for the crosstalk between the two signals. Based on these results, we suggest that XSufu exerts its diverse roles as a mediator and dual inhibitor of Hh/Gli and Wnt/β-catenin signaling.

XSufu is a dual regulator of Hh and Wnt signaling

In agreement with previous studies that have characterized Sufu as an essential regulator of mammalian Hh signaling (Cooper et al., 2005; Svärd et al., 2006; Taylor et al., 2002; Varjosalo et al., 2006), we show that XSufu is sufficient and required to reduce Hh target gene expression. A previous report challenged the concept of Sufu as exclusive regulator of Hh signaling and suggested instead that mammalian Sufu may directly inhibit Wnt signaling through a mechanism where Sufu binds to β-catenin and transports it out of the nucleus (Meng et al., 2001). In medulloblastoma patients a germline mutation in the human Sufu gene has been identified that prevents the protein from inhibiting Wnt signaling (Taylor et al., 2004). Yet whether Sufu-mediated suppression of Wnt signaling is relevant for embryonic development has remained elusive. We provide evidence that XSufu is a potent and critical inhibitor of Wnt/β-catenin signaling in *Xenopus*. First, overexpression of *XSufu* mRNA inhibits *in vitro* and *in vivo XWnt8*-induced target gene expression and dorso-anterior development. Then, antisense morpholino oligonucleotide (MO)-mediated knockdown of XSufu induces the direct Wnt targets *Siamois* and *Xnr3* in animal cap explants. Third, XSufu is sufficient and required to restrict endogenous expression of *Xnr3* and the Wnt-responsive *Chordin* gene in the early gastrula embryo. We note that the microcephaly observed in *XSufu*-morphant embryos may result from derepressing late Wnt signals that posteriorize the embryonic axis at more advanced stages of development. The suppression of the Wnt pathway by *XSufu* is not mediated through inhibition of Hh/Gli1 signals, as overexpression of *XGli1* fails to reverse the suppressive effect of *XSufu* on *XWnt8*-induced secondary axis induction and *XWnt3a*-induced posteriorization of the neural plate. Our observation that XSufu can be co-immunoprecipitated with Xβ-catenin suggests regulation through direct interaction instead. Previous studies did not detect a function of Sufu in inhibiting Wnt signaling in the mouse (Svärd et al., 2006; Varjosalo et al., 2006). Other Wnt pathway regulators may compensate for the loss of Sufu in knockout mice. In mouse embryonic fibroblasts, Gli1 or Gli2 proteins suppress Wnt signaling *via* transcriptional activation of the soluble Wnt antagonist sFRP1 (secreted frizzled-related protein-1, Katoh and Katoh, 2006; He et al., 2006), suggesting that increased levels of active Gli proteins in Sufu knockout mice may mask a possible role of Sufu in inhibiting Wnt signaling. It may be worthwhile to monitor Wnt signaling in mice with a compound knockout of Sufu, Gli1, and Gli2. Our study suggests that XSufu is a dual inhibitor of Hh and Wnt signaling

in the *Xenopus* embryo, and future studies are needed to carefully investigate a possible function of Sufu as negative regulator of Wnt signaling in mammalian development.

Roles of Hh, Wnt and XSufu in neural induction and eye specification

This study supports previous findings that overexpression of Hh ligands promotes neural fate in *Xenopus* embryos (Franco et al., 1999; Lai et al., 1995). Our observation that *XGli1* phenocopies the effect of *XBhh* mRNA suggests that Hh signals favor neural induction through activation of Gli transcription factors. The demonstration that inhibition of Hh signaling at the receptor (*XPtc1Δloop2*) or transcription factor level (*GLI3′CΔClaI*) impair with neural plate development point to a crucial role of the Hh/Gli pathway in this process. How Hh/Gli signals may contribute to neural induction remains an open question, and a possible integration with other neural inducing pathways needs to be further investigated. Our observation that late Wnt/β-catenin signals prevent formation of neural tissue is in agreement with a previous work (Heeg-Truesdell and LaBonne, 2006) and may occur through a mechanism involving sequestration of GSK3 in multivesicular bodies and sustained BMP/Smad1 signaling (Fuentealba et al., 2007; Taelman et al., 2010). In accordance with elevated expression levels of XSufu at the border of the neural plate, our functional experiments showed that XSufu is necessary and sufficient to restrict the neural plate marker *Sox2*. XSufu may exert its anti-neural function through inhibiting Hh/Gli1 signals. Alternatively, XSufu could regulate early pro-neural β-catenin signals. In the *Xenopus* blastula, dorsal β-catenin signals induce neural tissue *via* extinction of *BMP4* transcription (Baker et al., 1999) and the transcriptional activation of secreted BMP antagonists (Wessely et al., 2001). We identified XSufu as negative regulator of the organizer-specific *Chordin* and *Xnr3* genes. Chordin and Xnr3 are secreted BMP antagonists that induce neural differentiation (Hansen et al., 1997; Haramoto et al., 2004; Sasai et al., 1995), and a recent genome-wide screen in *Xenopus* found these genes as most strongly induced by maternal β-catenin signals (Wessely et al., 2004). We suggest that in the blastula embryo, XSufu may restrict Spemann′s organizer formation by reducing dorsal β-catenin signaling. Hence XSufu may impair with neural induction through inhibiting Hh/Gli and early Wnt/β-catenin signals.

In gain- and loss-of-function experiments, we could extend previous findings (Cornesse et al., 2005; Marine et al., 1997) and show that Hh and Wnt signals impair with the development of the eye field. Our temporal analysis revealed that an inducible Tcf construct (*THVGR*) can suppress eye fate when activated at the onset of neurulation. At this stage, XSufu is expressed in the eye field and continues to be abundant in the optic vesicles at later stages of embryogenesis. We show that overexpression of XSufu causes an expansion of the eye-specific marker *Rx2A* and enlargement of eye structures, while downregulation of XSufu has the opposite effect. We did not observe changes in cell proliferation and apoptosis suggesting that XSufu directly affects cell fate. The observation that in *XSufu*-morphant embryos suppression of the Hh or Wnt pathways restores normal *Rx2A* expression suggests that XSufu may represent a mechanism to terminate Hh and Wnt signaling in early eye anlage. A conditional knockout of Sufu in the mouse eye recently introduced a role of Sufu in maintaining multipotency of neural progenitor cells in the retina (Cwinn et al., 2011). Interestingly, Sufu-depleted retinal progenitor cells exhibited sustained expression of *Sox2* and downregulation of *Rax*/*Rx*, suggesting a conserved function of Sufu in the regulation of these transcription factors in the developing CNS of *Xenopus* and the mouse.

Biphasic model for XSufu in anteroposterior patterning

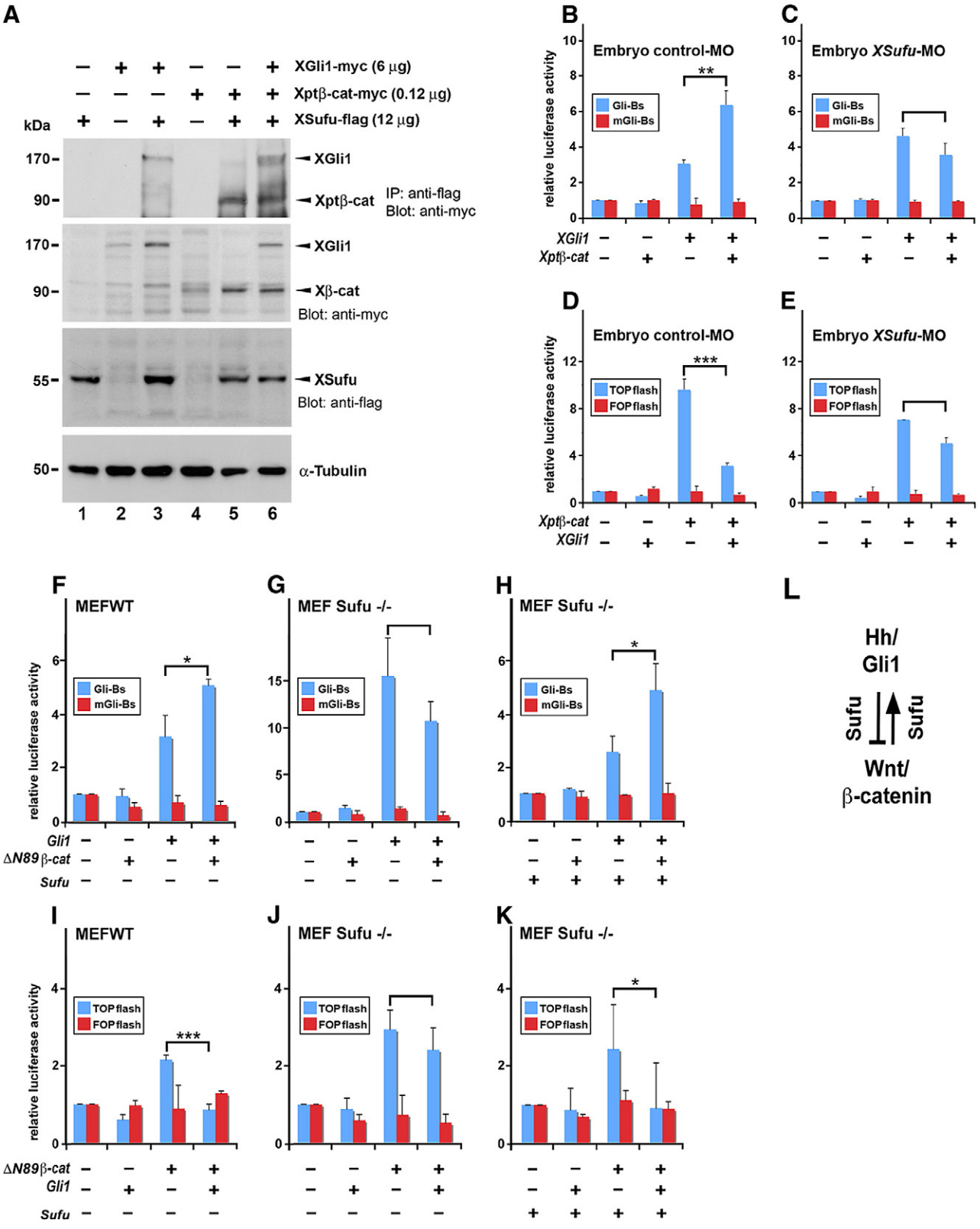
In loss-of-function experiments, depletion of XSufu by antisense morpholino oligonucleotides caused a posterior shift of the brain-specific markers *Otx2*, *En2* and *Krox20*. Similar results were obtained in gain-of-function experiments, with increasing doses of *XSufu* mRNA causing a proportional increase in the posteriorward shift of hindbrain rhombomeres. How can this apparent paradox be explained that up- and downregulation of one gene elicit the same response? In Fig. 9, we suggest a biphasic model for the role of XSufu in anteroposterior patterning of the developing CNS. The abundant expression of *XSufu* at the anterolateral margin of the neural plate suggests that under normal conditions XSufu interacts rather with anterior XGli proteins (Ekker et al., 1995; Lee et al., 1997; Marine et al., 1997) than with posterior Xβ-catenin (Kiecker and Niehrs, 2001). We propose that knockdown of XSufu may cause anteriorization through releasing active XGli proteins in the anterior neural plate. In contrast, ectopically expressed XSufu may suppress posterior fate by binding to and inhibiting Xβ-catenin in posterior locations of the neural plate. Regulation of the Wnt/β-catenin pathway by XSufu is supported by the rescue of normal anteroposterior pattern of brain markers upon overexpression of XSufu and simultaneous inhibition of Wnt/β-catenin signaling. Hence lowered XSufu concentration appears to stimulate anterior Hh/Gli signaling and high XSufu protein levels to downregulate posterior Wnt/β-catenin signaling, leading in each case to anteriorization of the CNS. Recent studies in mouse embryonic fibroblasts introduced a novel positive role of Sufu in regulating Hh signaling through promoting the stability of Gli2 and Gli3 full-length activators (Chen et al., 2009; Jia et al., 2009; Wang et al., 2010). Future studies will need to address the impact of XSufu on XGli protein stability and its relevance for pattern formation in the *Xenopus* embryo.

XSufu mediates the crosstalk between Hh and Wnt signals

Of particular interest is the interplay of Hh/Gli1 and Wnt/β-catenin signaling as well as the role that Sufu plays herein. We observed that *XGli1* counteracted *XWnt8*-induced secondary axis formation and *XWnt3a*-induced posteriorization of the neural plate. In mRNA-injected *Xenopus* embryos and mouse embryonic fibroblasts (MEFs), exogenous *Gli1* inhibited Wnt reporter activity under overexpression of *β-catenin*. A similar inhibition of Wnt/β-catenin transcriptional activity by Hh/GLI1 overexpression has been reported for human cancer cells (Akiyoshi et al., 2006; Yanai et al., 2008), and Indian Hedgehog has been described as a negative regulator of Wnt signaling in colonic epithelial cell differentiation

Fig. 8. Biochemical interaction and function of Sufu in the cross-talk of Hh/Gli1 and Wnt/β-catenin signaling. (A) XSufu-flag immunoprecipitates with XGli1-myc and Xptβ-catenin-myc in HEK293 cells 36 h after co-transfection. Proteins in cell lysates were analyzed by Western blot with antibodies against myc (for XGli1 and Xptβ-catenin), flag (for XSufu) and mouse Sufu. α-Tubulin was used as the loading control. (B–K) Luciferase reporter assays in lysates of *Xenopus* embryos at stage 10.5 following injection of morpholino oligonucleotides (MOs) and mRNAs of *Xptβ-catenin* and *XGli1*, using the *8xGli-Bs-luc* reporter to monitor Hh/Gli signaling (with *8x3′mGli-Bs-luc* as negative control) and the *TOP-flash* reporter to monitor Wnt/β-catenin signaling (with *FOP-flash* as negative control). Reporter (firefly) activities were normalized to pRL-TK (*Renilla*) activities to control for transfection/injection efficiencies. Error bars represent standard deviation from three representative experiments. Control embryos are injected with reporter plasmid + pRL-TK. (B,C) *Xptβ-catenin* upregulates the *XGli1*-induced Hh reporter in control-MO-, but not in *XSufu*-MO-injected embryos. (D,E) *XGli1* downregulates the *Xptβ-catenin*-induced Wnt reporter in control-MO-, but has no significant effect in *XSufu*-MO-injected embryos. (F–K) Hh and Wnt reporter assays in lysates of MEF WT, and MEF *Sufu*−/− cells 48 h after transfection with mouse *ΔN89β-catenin*, *Gli1* and *Sufu* cDNA plasmids. Control cells are transfected with empty vector. (F–H) *ΔN89β-catenin* promotes the *Gli1*-activated Hh reporter in MEF WT (F), but not in MEF *Sufu*−/− cells (G). Addition of *Sufu* cDNA restores the stimulating effect of *ΔN89β-catenin* on Gli1-induced Hh reporter activity (H). *Gli1* inhibits the *ΔN89β-catenin*-activated Wnt reporter in MEF WT (I), but has no significant effect in MEF *Sufu*−/− cells (J). Addition of *Sufu* cDNA rescues the inhibitory effect of *Gli1* on *ΔN89β-catenin*-induced Wnt reporter activity (K). Asterisks denote statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (L) Summary of protein interactions.

(van den Brink et al., 2004). SHH-N has been shown to inhibit Wnt signaling through transcriptional activation of *sFRP2* during somite development in the mouse (Lee et al., 2000). We found that the negative regulation of the canonical Wnt pathway by Hh/Gli1 signals depends on the presence of Sufu since in *XSufu*-morphant *Xenopus* embryos and *Sufu* knockout MEF cells, *Gli1* fails to block *β-catenin*-induced Wnt reporter activity, and addition of exogenous *Sufu* to MEF *Sufu*−/− cells could restore the inhibitory effect of *Gli1* on the Wnt/β-catenin response. We also observed that exogenous *β-catenin* stimulated Hh reporter activity under overexpression of *Gli1* in *Xenopus* embryos and MEFs. Similarly, *β-catenin* enhances *Gli1*-transcriptional activity in human cancer cells in a TCF/LEF-independent manner (Maeda et al., 2006). We noted that in *XSufu*-deficient *Xenopus* embryos and *Sufu*-depleted MEF cells, *β-catenin* did



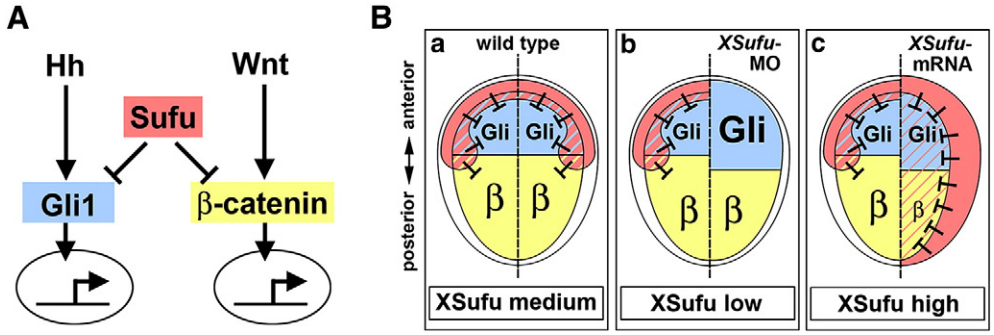


Fig. 9. Sufu as a dual repressor of Hh/Gli and Wnt/β-catenin signaling. (A) Interactions of XSufu with Gli1 and β-catenin. (B) Biphasic model for regulation of anteroposterior patterning by XSufu in late *Xenopus* gastrulae. (a) Under normal conditions, XSufu at the anterolateral margin of the neural plate suppresses anterior Gli and less efficiently posterior β-catenin activity. (b) Depletion of XSufu derepresses anterior Gli activity, which causes excessive anterior neural induction. (c) Ectopic expression of XSufu suppresses posterior β-catenin activity and prevents posteriorization of the neural plate.

not further stimulate *Gli1*-induced Hh reporter activity unless exogenous *Sufu* was added, suggesting that the levels of endogenous Sufu protein may be critical for the positive effects of Wnt/β-catenin signals on the Hh/Gli pathway. The mechanism of how Sufu may facilitate the inhibition of Wnt/β-catenin signaling by Gli1 and the activation of Hh/Gli1 response to β-catenin is not understood and needs to be addressed in future studies. In conclusion, Sufu acts as a dual regulator and may have an integrating role in linking Hh/Gli and Wnt/β-catenin signaling in the *Xenopus* embryo.

Materials and methods

Injections of RNA, DNA, and morpholino oligonucleotides

Sense RNA for microinjection was synthesized using the mMessage Machine kit (Ambion). DNA templates were linearized, transcribed and mRNAs injected per blastomere as follows: pXEX-*nlacZ* (*Xba*I, T7, 100 pg), pT7TS-*XBhh* (*Bam*HI, T7, 500 pg; Ekker et al., 1995), pCS-*XGli1-myc* (*Not*I, Sp6, 150 pg; Lee et al., 1997), pCS-*XPtc1ΔLoop2* (*Not*I, Sp6, 1 ng; Koebernick et al., 2003), pCS-*Gli3C′ΔClaI-myc* (*Not*I, Sp6, 100 pg; Ruiz i Altaba, 1999), pSP64T-*XWnt8-myc* (*Bam*HI, Sp6, 12.5 pg; Christian and Moon, 1993), pCS-*dnXWnt8* (*Asp*718, Sp6, 100 pg; Hoppler et al., 1996), pCS-*XSufu* (*Not*I, Sp6, 750 pg), pCS-*XSufu** (*Not*I, Sp6, 300 pg), pCS-*Xptβ-catenin-HA* (*Not*I, Sp6; Yost et al., 1996), and pCS-*THVGR* (*Not*I, Sp6, 20 pg; Wu et al., 2005). pCS-*XWnt3a* DNA (Saint-Jeannet et al., 1997) was injected at 30 pg per blastomere. The *XSufu*-MO (CTT CCA AGC AAA TCA CCA ACA GGA G) and standard control-MO were obtained from Gene Tools LLC and injected at 25 ng per blastomere.

Embryo manipulations and RT-PCR

X. laevis embryos and explants were obtained, cultured, microinjected and subjected to whole-mount *in situ* hybridization and lineage tracing as described (Hou et al., 2007). Gelatin/albumin sections (40 μm) were done using a Leica VT1200S vibratome. RT-PCR assays were as reported (Hou et al., 2007). For gene-specific primers and PCR conditions, see Supplemental experimental procedures.

Western blot analysis

Western blots were performed with monoclonal antibodies against c-myc (1:2000; Santa Cruz, sc-47694), α-Tubulin (1:1000; Sigma, T9026), anti-flag-HRP conjugate (1:1000; Sigma, A8592), and a polyclonal antibody against mouse Sufu (1:5000; Kise et al., 2009). For immunoprecipitation and additional Western blot methods, see Supplemental experimental procedures.

Reporter luciferase assays

NIH3T3, MEF WT and MEF *Sufu*−/− cells (Svärd et al., 2006) were transfected in 6-well plates with Fugene HD (Roche), 0.8 μg reporter constructs and the following plasmid DNAs: pCS-*XGli1-myc* (0.2 μg; Lee et al., 1997), pCS-*Xptβcatenin-HA* (0.1 μg; Yost et al., 1996), pCS2-*XSufu-Flag* (0.5 μg), pCDNA3-*mGli1-His* (2 μg; Sasaki et al., 1999), pCDNA3-*mΔ89β-catenin* (2 μg; Meng et al., 2001), pCMV5-*mSufu-myc* (0.5 μg; Ding et al., 1999), pRL-TK (2.5 ng, Promega), and empty pCS2+. Embryos were animally injected at the 2-cell stage with 50 ng MOs and at the 4-cell stage with 400 pg *Xptβ-catenin-HA* mRNA, 2 ng *XGli1-myc* mRNA, 100 pg reporter construct, and 10 pg pRL-TK. Luciferase activities were measured using the Dual luciferase reporter assay system (Promega).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.07.035.

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