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Tbx2 regulates anterior neural specification by repressing FGF signaling pathway

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ABSTRACT

During early embryogenesis, FGF signals regulate the antero-posterior (AP) patterning of the neural plate by promoting posterior cell fates. In particular, BMP signal-mediated attenuation of FGF pathway plays a critical role in the determination of the anterior neural region. Here we show that Tbx2, a T-box transcriptional repressor regulates anterior neural specification by suppressing FGF8 signaling pathway in *Xenopus* embryo. *Tbx2* is expressed in the anterior edge of the neural plate in early neurulae. Overexpression and knockdown of *Tbx2* induce expansion and reduction in the expression of anterior neural markers, respectively. It also suppresses FGF8-induced ERK phosphorylation and neural caudalization. Tbx2, which is a target gene of BMP signal, down-regulates FGF8 signaling by inhibiting the expression of Flrt3, a positive regulator of this pathway. We found that Tbx2 binds directly to the T-box element located in the promoter region of *Flrt3* gene, thereby interfering with the activity of the promoter. Consistently, Tbx2 augmentation of anterior neural formation is inhibited by co-expression of *Flrt3*. Furthermore, disruption of the anterior-most structures such as eyes in Tbx2-depleted embryos can be rescued by inhibition of Flrt3 function or FGF signaling. Taken together, our results suggest that Tbx2 mediates BMP signal to down-regulate FGF signaling pathway by repressing *Flrt3* expression for anterior tissue formation.

1. Introduction

During vertebrate early development, neural induction, which generates the neural plate, is followed by neural patterning that confers regional specificity on the neural ectoderm along the anterior-posterior (A-P) and dorso-ventral (D-V) axes. Nieuwkoop proposed the "activation-transformation" model in which neural tissue is initially induced with anterior identity by the organizer (activation) and subsequently caudalized by additional signals from the mesoderm (transformation) (Sasai and De Robertis, 1997). Work in *Xenopus* revealed that inhibition of BMP signaling is sufficient to induce neural differentiation from naïve ectodermal explants (Munoz-Sanjuan and Brivanlou, 2002). Thus, neural induction requires bone morphogenetic protein (BMP) antagonists such as Noggin, Chordin and Follistatin, from the Spemann's organizer region of the embryo to induce neural fate (De Robertis and Kuroda, 2004; Harland, 2000). Fibroblast growth factor (FGF) signal has also been proposed to be required for neural induction in chick and frog embryos (Delaune et al., 2005; Launay et al., 1996; Streit et al., 2000; Wilson et al., 2000). These disparate observations of neural induction have been reconciled through a model by which the activation of FGF signaling promotes neural induction at the expense of epidermal fates through the MAPK-mediated phosphorylation of the linker region of BMP-sensitive Smads, which leads to degradation of the Smads and suppression of BMP signaling pathway (De Robertis and Kuroda, 2004; Pera et al., 2003; Sapkota et al., 2007).

Wnt, FGF, Nodal and retinoic acid signals have been shown to have caudalizing activities when combined with the neural inducers (Rallu et al., 2002). Although the neural tissue induced by Nieuwkoop's activation step is of the forebrain character, anterior neuroectoderm specification requires the active roles of inhibitors of these posteriorizing signals from the organizer. Dkk-1, Lefty, Frzb-1, Cerberus and Del1 contribute to forebrain specification by restricting the activities of Wnt and Nodal signaling in a non-cell autonomous manner (Bouwmeester et al., 1996; Glinka et al., 1998; Leyns et al., 1997; Takai et al., 2010). Intrinsic factors such as XsalF, Six3, Tcf3 and Hesx1 also play critical roles in maintaining anterior forebrain identity

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by attenuating cellular responsiveness to Wnt signaling (Andoniadou et al., 2011; Lagutin et al., 2003; Onai et al., 2004). An endoplasmic reticulum (ER) protein, Shisa, specifies forebrain tissue by inhibiting the post-translational maturation and trafficking to the cell surface of FGF receptor, which leads to down-regulation of FGF signaling (Yamamoto et al., 2005). Late activation of BMP signal in the anterior neural plate also functions to maintain forebrain character by attenuating FGF signaling pathway *via* repression of *Flrt3* expression (Cho et al., 2013). However, the downstream effectors mediating the BMP inhibition of FGF pathway during anterior neural formation remains unknown.

Tbx2 is a member of the Tbx2 subfamily of T-box transcription factors, which includes the closely related Tbx3, Tbx4 and Tbx5 genes (Abrahams et al., 2010). These members have the DNA-binding domain known as the T-box and recognize the core sequence GGTGTGA, referred to as the T-element, as a monomer. Unlike most members of the T-box family, Tbx2 functions as a transcriptional repressor, which is mediated by the repressor domain in the C-terminal. In human and mouse, Tbx2 is expressed in a wide variety of tissues including heart, kidney, lung, ovary, and spleen. Thus, it has been shown to play crucial roles in specifying the posterior digit identity (Suzuki et al., 2004), patterning the atrioventricular canal of the heart (Harrelson et al., 2004) and defining the territory of the embryonic kidney (Cho et al., 2011). Tbx2 also regulates embryonic development of the eye, brain, bone, mammary gland and melanocyte (Abrahams et al., 2010). Besides its key roles in development, Tbx2 is shown to be implicated in cell cycle regulation and amplified in a subset of cancers including breast, melanoma, liver and pancreatic, suggesting its role in tumorigenesis (Abrahams et al., 2010; Lu et al., 2010).

In this study, we have identified the novel role of Tbx2 in anterior forebrain specification in *Xenopus* early development. Although the distribution of Tbx2 transcripts in several tissues such as heart, pronephros, trigeminal ganglia, cement gland, and proctodeum has been previously described in *Xenopus* embryo (Hayata et al., 1999; Showell et al., 2006), its expression in the anterior neuroectoderm, which recapitulates the pattern of BMP signaling activity during the late gastrula and early neural stages (Cho et al., 2013), has gone unnoticed. Interestingly, we found that Tbx2 regulates anterior forebrain specification by mediating BMP antagonism of FGF signaling pathway. To do this, Tbx2, a target gene of BMP signal, represses directly the expression of fibronectin-leucine-rich transmembrane protein 3 (*Flrt3*) by binding to its promoter. Flrt3 regulates positively FGF signaling through MAP kinase pathway during cell adhesion, limb development and neuron guidance (Bottcher et al., 2004; Ogata et al., 2007; Tomas et al., 2011; Yamagishi et al., 2011). Therefore, we suggest a novel mechanism underlying the cross-talk between the BMP and FGF signaling pathways in vertebrate neural patterning.

2. Materials and methods

2.1. Embryo manipulation and chemical treatments

In vitro fertilization, microinjection and embryo culture were performed as described previously (Cho et al., 2011). Developmental stages of embryos were determined according to the Nieuwkoop and Faber's normal table of development (Nieuwkoop and Faber, 1994). For the induction of human glucocorticoid receptor ligand binding domain (hGR)-fused constructs, dexamethasone (DEX; 10 μM, Sigma) was added at the indicated time. SU5402 (20 μM, Calbiochem) was treated at the indicated stages to block FGF signaling.

2.2. Plasmid constructs and morpholino oligonucleotides

The open reading frame of *Xenopus laevis Flrt3* (accession No. NM_001095561) was amplified by PCR and inserted into the *BamHI*/*XhoI* sites of pCS2+ vector to generate *Flrt3*-CS2. The following constructs were described previously: *Tbx2*, *Tbx2ΔC-GR* and *VP16-Tbx2-GR* (Cho et al., 2011); FGF8 (Fletcher et al., 2006); Noggin (McGrew et al., 1997); Chordin (Sasai et al., 1994); BMP4 (Maeno et al., 1994); DN BMP4 receptor (tBR) (Suzuki et al., 1994). The antisense morpholino oligos (MOs) were purchased from Gene Tools. The sequences of MOs are as follows; *Tbx2* MO, 5′-GGAAAGCTGGATCTCTCATCGGTGC-3′; *Flrt3* MO-a, 5′-AATATCCAGTAGGCAGAAAGCAGCC-3′; *Flrt3* MO-b, 5′-GTTTCTGTAGACATGGTCACTGATG-3′. For complete knockdown of *Flrt3*, equal amounts of *Flrt3* MO-a and *Flrt3* MO-b were mixed and microinjected. Control (CO) MO was a standard morpholino oligo from Gene Tools whose sequence is 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

2.3. Lineage tracing and in situ hybridization

For lineage tracing, β-galactosidase mRNA (β-gal, 100 pg) was co-injected and its activity was visualized with Red-Gal substrate (Sigma). Whole-mount *in situ* hybridization was performed as described in (Harland, 1991). Anti-sense digoxigenin-labelled RNA probes were *in vitro* synthesized using template cDNA encoding *Tbx2* (Cho et al., 2011), *Flrt2* (Bottcher et al., 2004), *Flrt3* (Bottcher et al., 2004), *Otx2* (Blitz and Cho, 1995), *Krox20* (Bradley et al., 1993) and *Bf1* (Kiecker and Niehrs, 2001).

2.4. Western blotting

Western blotting was performed according to the standard protocol using p44/42 MAPK (Erk1/2) (1:1000, Cell signaling) and phospho-p44/42 MAPK (Erk1/2) (1:1000, Cell signaling) antibodies. Measurement of the intensities of Western blot bands was performed using ImageJ 1.40 g software. Statistical significance was determined using T-test using Microsoft Excel software. A p value of less than 0.05 was considered as significant.

2.5. RT-PCR

For RT-PCR analysis, total RNA was extracted from whole embryos and tissue explants using TRI Reagent (Molecular Research Center) and treated with RNase-free DNase I to remove genomic DNAs. RNA was transcribed using M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. PCR products were analyzed on 2% agarose gels. The numbers of PCR cycles for each primer set were determined empirically to maintain amplification in the linear range. The sequences of PCR primers used here are as follows: Flrt2 and Flrt3 (Bottcher et al., 2004); Tbx2 (Cho et al., 2011); Otx2, HoxB9, Krox20 and ODC (Choi and Han, 2005).

2.6. Promoter cloning and luciferase reporter assay

A genomic DNA fragment (2 kb) upstream of transcription initiation site of human *Flrt3* was amplified using the following primers: forward, 5′-CATGGTGTTTAATTCAAAGTGACACA-3′; reverse, 5′-GGTCAGCAGTGTTGAGGTCTTTATAC-3′. Genomic DNA sequence of *Xenopus Flrt3* was provided by Harland, R.M. and analyzed up to 3 kb upstream of its start codon. Wild-type promoter region of human *Flrt3* was subcloned into luciferase reporter plasmid pGL3 (pGL3-wt FLRT3pro-luc). Both of the T-box binding sites in this reporter gene were mutated by site-directed mutagenesis as shown in Fig. 4A to generate pGL3-mut FLRT3pro-luc construct. Injected embryos were lysed at late gastrula stages and luciferase activity was measured with a dual luciferase assay system (Promega). pRL-TK Renilla vector was used as an internal control. At least, three independent experiments were performed.

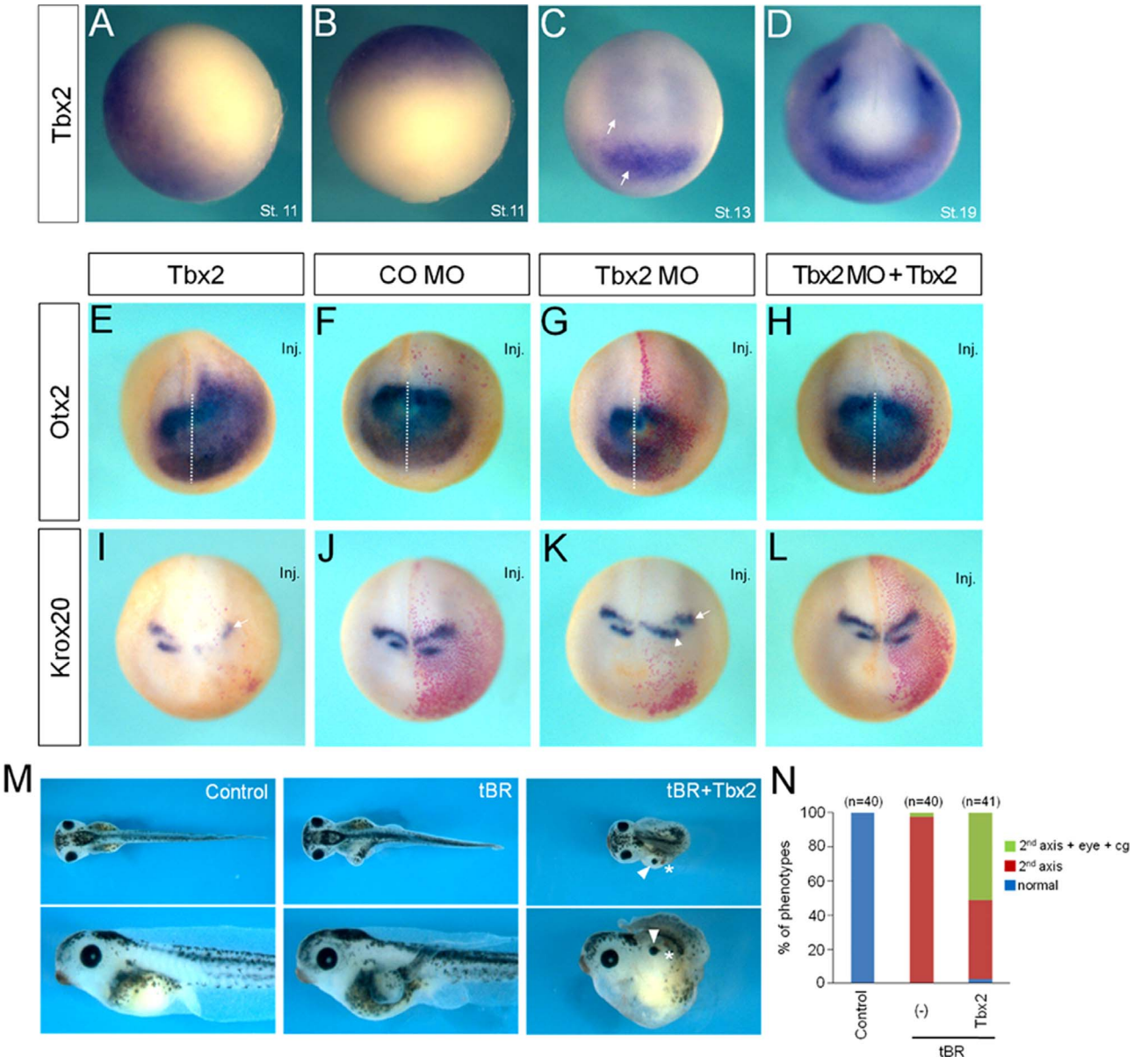


Fig. 1. Tbx2 is critical for anterior neural development. (A-D) Whole-mount *in situ* hybridization analysis showing the expression pattern of *Tbx2* at the gastrula and neurula stages. (A) Lateral view, anterior to the left. (B) Dorsal view, anterior to the top. (C, D) Anterior view, dorsal to the top. (E-L) Four-cell stage embryos were injected dorso-animally with *Tbx2* (200 pg), *Tbx2* MO (10 ng) and CO MO (10 ng) as indicated, cultured until stage 16–17 and then subjected to *in situ* hybridization against *Otx2* or *Krox20*. *LacZ* RNA (100 pg) was co-injected as a lineage tracer. All embryos are shown in anterior view with dorsal to the top. Inj. denotes the injected side of embryo. (M) For axis duplication assays, embryos were injected in the ventral region with *tBR* (100 pg) alone or with *Tbx2* (200 pg) and cultured to tadpole stages. Arrowheads and asterisks indicate eye and cement gland, respectively. (N) Quantification of axis duplication assays shown in (M). n, the total number of embryos analyzed. 2 nd axis, a partially duplicated axis; eye, a secondary eye; cg, a secondary cement gland; normal, normal embryos with single body axis.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed using HEK 293 T cells and Pierce Agarose ChIP Kit (Thermo Scientific) according to the manufacturer's instructions. For ChIP assay, Tbx2 (ab33298, Abcam) antibody, pGL3-wt FLRT3pro-luc and pGL3-mut FLRT3pro-luc constructs were used. PCR primers used for analysis of Tbx2 binding are as follows: P1 primers (flanking the upstream T-box binding site), forward, 5′-ACTTTCACACAGAAACTCTATCAGAAAGA-3′ and reverse, 5′-TCCTACCTTA TTGAACGATGTGAGTAAAT-3′; P2 primers (flanking the downstream T-box binding site), forward, 5′-GAGTATGAAGAGAATAGGTATTTTTATGAC-3′ and reverse, 5′-AGTATCTGTCCT AAATCCATTTGATTTGA-3′. As a control, primers against the region between both the T-box binding sites were used: CP forward, 5′-AGTCCCAGTCCCTCACCAGGAA-3′ and reverse, 5′-ACC TCATAACGTGTTGTCACAGTGC-3′.

3. Results

3.1. Tbx2 is essential for the formation of anterior brain tissue

In situ hybridization analysis showed that *Tbx2* is expressed in the ventral ectoderm and the prospective anterior neuroectoderm but not in the posterior neuroectoderm at the late gastrula stages (Fig. 1A and B). During early neurulation, its strong expression was detectable in the anterior edge of the neural plate and its less intense message in the anterior neural plate (Fig. 1C; arrows). Later, its transcripts became restricted to the cement gland and placodal area (Fig. 1D). This

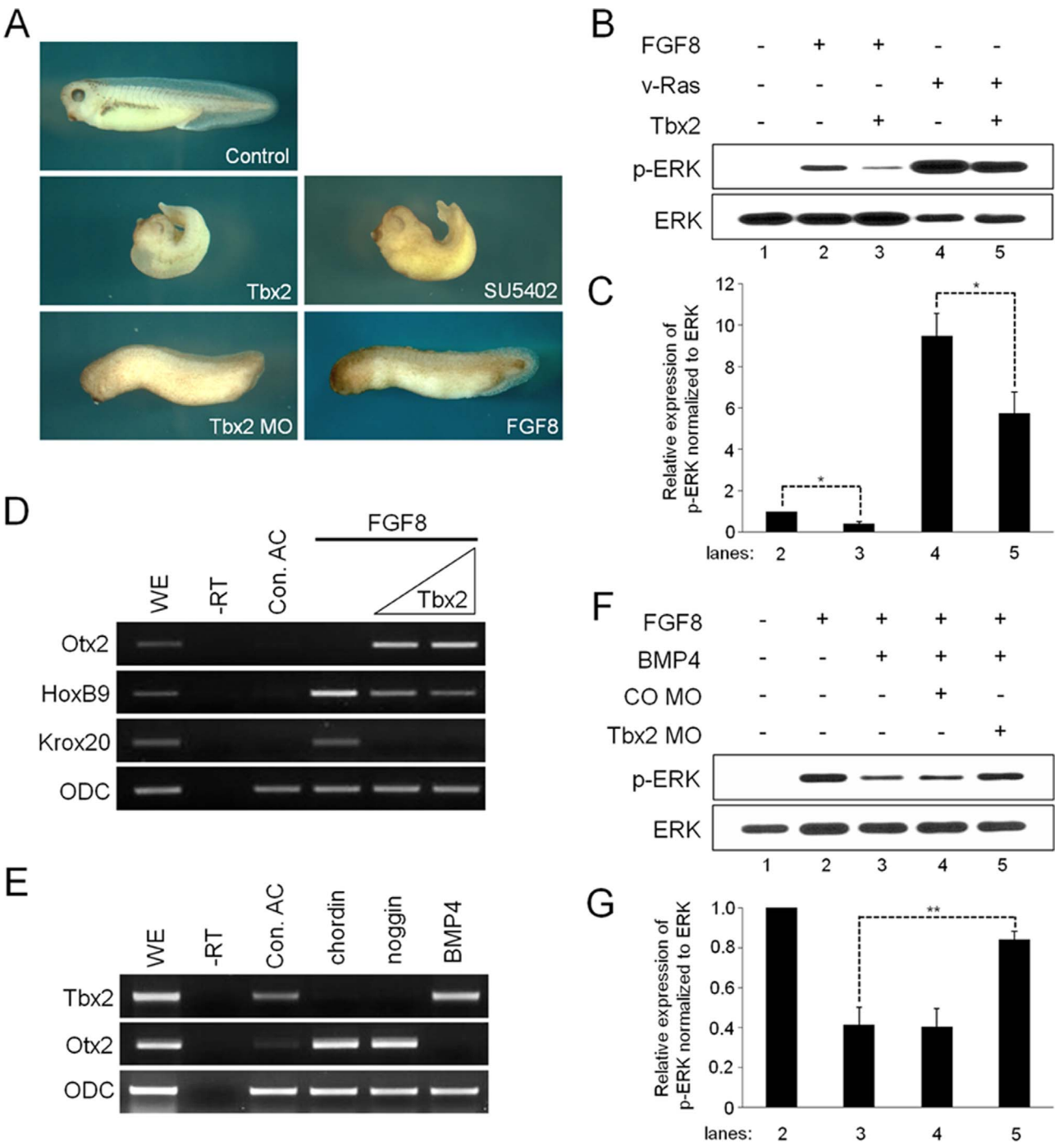


Fig. 2. Tbx2 acts as a target gene of BMP pathway to inhibit FGF signaling. (A) Phenotypes of embryos injected animally with *Tbx2* (200 pg), *Tbx2*MO (10 ng) or *FGF8* (200 pg) or treated with SU5402 (20 μM) from the four-cell stage to tadpole stage. Control, an uninjected control embryo. (B-G) Animal caps from the embryos injected animally with the indicated combination of *FGF8* (1 ng), *v-Ras* (50 pg), *Tbx2* (B, 200 pg; D, 100–200 pg), *chrodin* (200 pg), *noggin* (10 pg), *BMP4* (200 pg), *Tbx2* MO (10 ng) and CO MO (10 ng) were cultured to stage 12 for western blotting (B, C, F, G) and to stage 15 for RT-PCR analysis (D, E). (C) and (G) show quantification of the levels of p-ERK (normalized to ERK) for (B) and (F), respectively. Error bars indicate the standard error (SE) from three independent experiments. Asterisks above the bars denote **p*-value < 0.05 and ***p*-value < 0.01. ODC serves as a loading control. WE, whole embryo. Con. AC, uninjected control animal caps. −RT, a control in the absence of reverse transcriptase.

expression pattern of *Tbx2* suggests its possible role in anterior neural specification.

To test this assumption, we examined the effect of the gain- or loss-of-Tbx2 function on the expression of neural markers. Overexpression of *Tbx2* induced an obvious expansion in the expression of an anterior neural marker, *Otx2* (92%, n=42; Fig. 1E). Conversely, morpholino-mediated knockdown of *Tbx2* caused reduction of the anterior neural marker (62%, n=43; Fig. 1G), but control MO had no effect on it (Fig. 1F). This MO effect was recovered by co-injection of *Tbx2* RNA that is resistant to MO inhibition (93%, n=43; Fig. 1H), indicating the specificity of the MO effect. In addition, the expression of a posterior neural marker, *Krox20* in rhombomere 3 was highly repressed by Tbx2 (85%, n=45; Fig. 1I) but its residual expression was observed in rhombomere 5 (Fig. 1I, arrow). *Tbx2* MO caused an expansion of *Krox20* in rhombomere 3 (76%, n=48; Fig. 1K, arrowhead), which was reversed by co-injection of *Tbx2* RNA (Fig. 1L). Of note, its expression in rhombomere 5 was largely unaffected in *Tbx2*-depleted embryos (Fig. 1K, arrow). Given the different sensitivities of *Krox20* expression

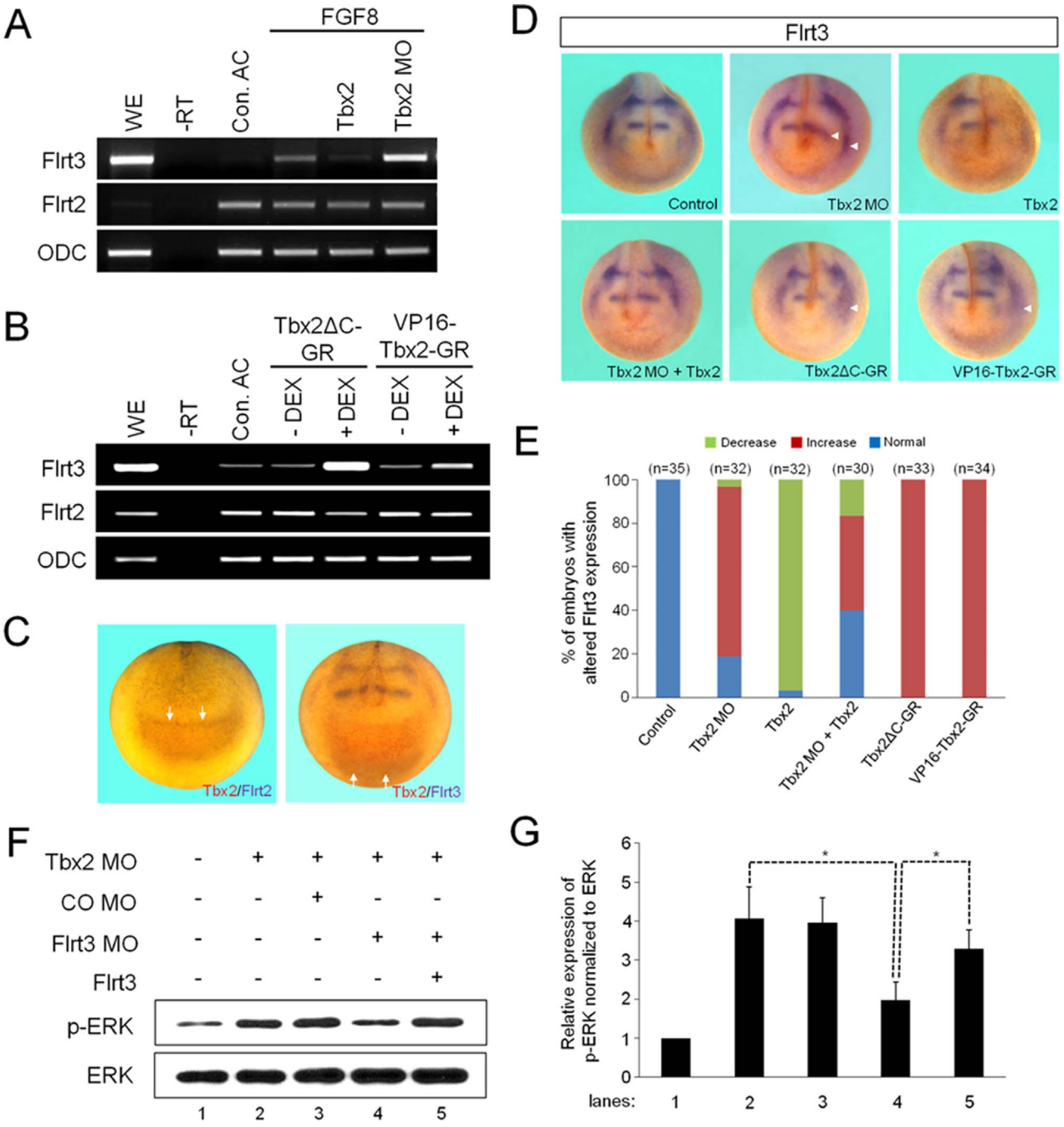


Fig. 3. Tbx2 down-regulates FGF signaling by repressing *Flrt3* expression. (A, B) Four-cell stage embryos were injected in the animal pole region as indicated with *FGF8* (1 ng), *Tbx2*(200 pg), *Tbx2* MO (10 ng), *Tbx2ΔC-GR* (200 pg) and*VP16-Tbx2-GR* (200 pg), and animal cap explants were dissected at stage 9, cultured until stage 12 in the absence or presence of dexamethasone (DEX; 10 μM) as indicated and then subjected to RT-PCR analysis. (C) Double *in situ* hybridization comparing the expression pattern of *Tbx2* (red) with that of *Flrt3* or *Flrt2* (purple) in mid-neurulae. (D) *Flrt3* expression in embryos injected dorso-animally with *Tbx2* (200 pg), *Tbx2* MO (10 ng), *Tbx2ΔC-GR* (200 pg) and *VP16-Tbx2-GR* (200 pg) as indicated. Embryos injected with *Tbx2ΔC-GR* or *VP16-Tbx2-GR* were treated with DEX from stage 12–17. Arrowheads denote expansion of *Flrt3* expression. In (C) and (D), embryos are shown in anterior view with dorsal to the top. (E) Quantification of the experiments shown in (D). (F) VMZ explants from the embryos injected ventrally with *Tbx2* MO (30 ng), CO MO (30 ng), *Flrt3* MO (30 ng) and *Flrt3* (200 pg) as indicated were subjected to western blotting. (G) Quantification of the levels of p-ERK (normalized to ERK) from three independent experiments for (F). Error bars indicate the standard error (SE). *p-value < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in rhombomeres 3 and 5 to the levels of inducing signals such as Wnt and FGF (Kiecker and Niehrs, 2001; Walshe et al., 2002), these results suggest that Tbx2 might affect the signals critical for anteroposterior patterning of neural plate.

We also tested the anteriorizing effect of *Tbx2* in a secondary axis assay. Inhibition of BMP signaling in the ventral region of *Xenopus* embryo induces an incomplete secondary dorsal axis without a head structure (De Robertis et al., 2000). Since the gain-of-Tbx2 function up-regulates the expression of the anterior neural marker as shown above, we investigated whether it could induce anterior structures including eyes and cement gland in such a partial secondary axis. Ventral injection of a dominant negative BMP receptor (*tBR*) induced a secondary axis without such anterior-most structures (Fig. 1M and N). Interestingly, co-injection of *tBR* and *Tbx2* caused a significant increase in formation of a duplicated axis with eyes and a cement gland (Fig. 1M and N). Overall, these results suggest a critical role of *Tbx2* in formation of anterior structures.

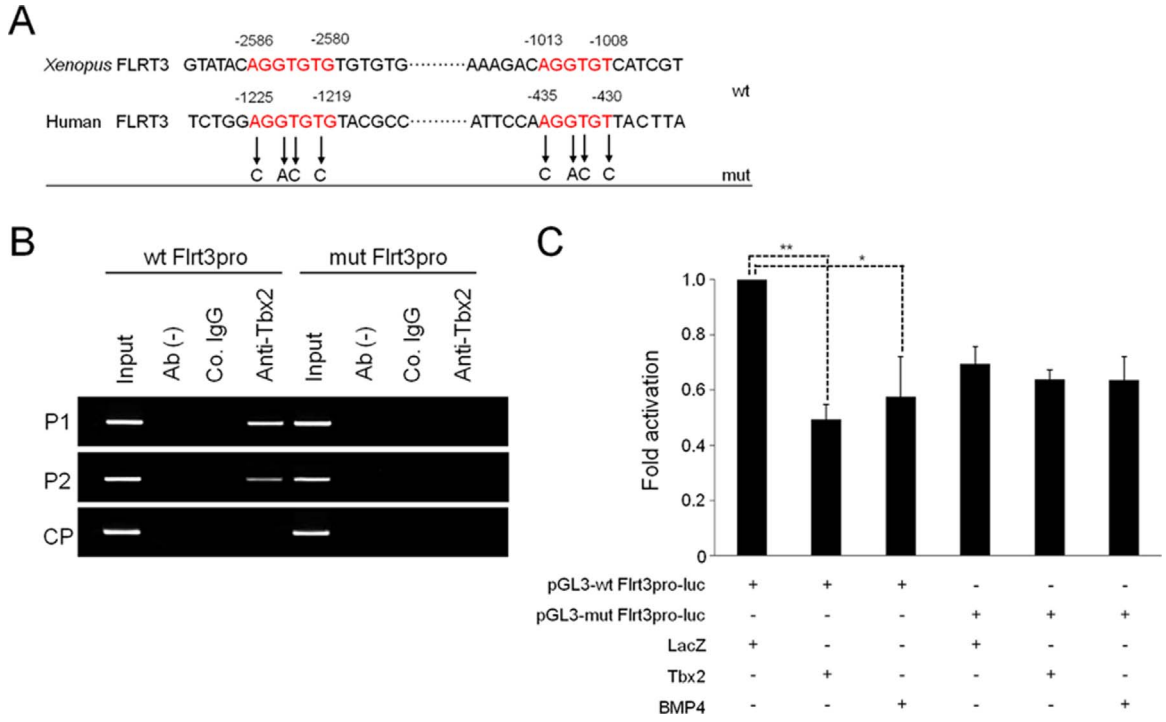


Fig. 4. Tbx2 binds to the promoter of *Flrt3* gene. (A) Alignment of wild-type (wt) putative T-box binding sites (in red) in the promoter regions of *Xenopus* and human *Flrt3* and the sequence of the mutant (mut) binding sites. (B) Chromatin immunoprecipitation (ChIP) analysis using HEK 293 T cells transfected with human Tbx2 along with DNA constructs harboring wild-type or mutant T-box binding sites of human *Flrt3*. P1, P2 and CP denote primers used for PCR reactions (Materials and Methods). Input, promoter DNA in cross-linked DNA-protein mix before immunoprecipitation. Ab (-), immunoprecipitation without antibody. Co. IgG, control rabbit IgG. (C) Suppression of the activity of *Flrt3* promoter-driven reporter by Tbx2 or BMP4 requires T-box binding sites. Embryos were injected dorsally with pGL3-wt Flrt3pro-luc (40 pg), pGL3-mut Flrt3pro-luc (40 pg), LacZ (200 pg), Tbx2 (200 pg) and BMP4 (200 pg) as indicated and incubated until stage 11, when firefly and Renilla luciferase activities were determined. Each experiment was carried out in triplicate and error bars indicate the standard error (SE) from three independent experiments. **p*-value < 0.05 and ***p*-value < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Tbx2 mediates BMP signal to inhibit FGF pathway

Overexpression of *Tbx2* caused loss of axial tissues from posterior to anterior in embryos (Fig. 2A), which is similar to the phenotype of embryos treated with SU5402, a blocker of FGF signaling. Like overexpression of *FGF8*, knockdown of *Tbx2* impaired head formation (Fig. 2A). These morphological phenotypes indicate an antagonistic relationship between Tbx2 and FGF signaling pathway. Thus, we investigated whether Tbx2 interferes with FGF signaling. As shown in Fig. 2B and C, *Tbx2* inhibited significantly ERK phosphorylation induced by *FGF8* or *v-Ras*, a downstream critical effector of FGF signaling. Consistently, co-injection of *Tbx2* reversed *FGF8* induction of neural tissue of a posterior character, thereby down-regulating the expression of posterior neural markers, *Krox20* and *HoxB9* and up-regulating that of an anterior neural marker, *Otx2*, respectively, in the *FGF8*-stimulated animal cap cells (Fig. 2D). *Tbx2* expression was increased by *BMP4* and completely inhibited by BMP antagonists such as *chordin* and *noggin* in the animal cap tissue (Fig. 2E), suggesting that *Tbx2* is a target gene of BMP4 signal. We have previously shown that BMP signal regulates anteroposterior neural patterning by attenuating FGF signaling pathway (Cho et al., 2013). Given the above data, it is possible that Tbx2 could mediate this BMP inhibition of FGF signaling. In support of this, *BMP4* could not inhibit *FGF8* induction of ERK phosphorylation in the animal cap cells depleted of *Tbx2* (Fig. 2F and G, lanes 3 and 5). Collectively, these results suggest that Tbx2 functions as a mediator of BMP signal to attenuate FGF signaling pathway during the anteroposterior neural patterning.

3.3. Tbx2 down-regulates Flrt3 expression

BMP inhibition of FGF signaling has been shown to involve repression of the expression of *Flrt3*, a positive regulator of the latter pathway (Cho et al., 2013). Therefore, we wanted to determine whether *Tbx2*, a transcriptional repressor, is responsible for the down-regulation of *Flrt3* expression. Injection of *FGF8* induced *Flrt3* expression in the animal caps, which could be inhibited by co-injection of *Tbx2* and further enhanced by its depletion (Fig. 3A). In addition, we generated dexamethasone (DEX)-inducible forms of *Tbx2*, which might activate rather than repress transcription, by deleting the C-terminal repressor domain of *Tbx2-GR* (*Tbx2ΔC-GR*) or fusing the VP16 activator domain to *Tbx2-GR* (*VP16-Tbx2-GR*) (Cho et al., 2011) and examined their effects on *Flrt3* expression. Unlike wild-type *Tbx2*, these modified forms of *Tbx2* up-regulated *Flrt3* expression in a DEX-dependent manner (Fig. 3B). In contrast, *Flrt2* expression was marginally affected by overexpression and depletion of *Tbx2*. Since Tbx2 has distinct *in vitro* effects on the respective expression of *Flrt2* and *Flrt3*, the expression pattern of *Tbx2* was compared with that of *Flrt2* or *Flrt3* in neurulae by double *in situ* hybridization. *Tbx2* was expressed in crescent form in the anterior edge of mid-neurulae (Fig. 3C). *Flrt3* expression did not largely overlap with that of *Tbx2*; in particular, the crescent positive for *Tbx2* expression appeared to be closely apposed to *Flrt3*-expressing anterior neural ridge (arrows). Notably, *Flrt2* expression (arrows) was detectable in the uppermost part of the area expressing *Tbx2* messages as shown in (Bottcher et al., 2004). These expression patterns are in line with the effect of Tbx2 on the expression of *Flrt2* or *Flrt3*.

We further investigated whether the *in vivo* expression of *Flrt3* in the anterior neural tissue might be affected by the gain- and loss-of-function of *Tbx2*. Knockdown of *Tbx2* led to expansion of *Flrt3* expression in the anterior brain region (78%, n=32; Fig. 3D and E), which was reversed by co-injection of *Tbx2*. In contrast, overexpression of *Tbx2* strongly inhibited *Flrt3* expression in the anterior area (96.8%, n=32; Fig. 3D and E). Like *Tbx2* MO, injection of *Tbx2ΔC-GR* or *VP16-Tbx2-GR* resulted in a marked increase in *Flrt3* expression in the

anterior neural tissue (Fig. 3D and E). Furthermore, we tested whether Tbx2 repression of *Flrt3* expression is relevant to its attenuation of FGF signaling. As shown in Fig. 3F and G, depletion of *Tbx2* augmented ERK phosphorylation in the ventral marginal zone (VMZ) tissue where *Tbx2* expression is abundant (lanes 1 and 2), which is reversed by co-injection of *Flrt3* MO but not by CO MO (lanes 3 and 4). This effect of *Flrt3* MO was abrogated by co-injection of *Flrt3* mRNA resistant to MO inhibition (lane 5). Taken together, these results suggest that *Tbx2* functions as a repressor of *Flrt3* expression to inhibit FGF signaling pathway.

3.4. *Flrt3* is a direct target gene of *Tbx2*

We further investigated whether *Tbx2* represses directly or indirectly *Flrt3* expression. Analysis of the promoter regions of human and *Xenopus Flrt3* revealed that there are two consensus T-elements (AGGTGT and AGGTGTG) in both of them which are putative T-box binding sequences, though they do not match exactly the conserved T-element sequence (AGGTGTGA). These elements reside at −430 to −435 and −1219 to −1225 base pairs upstream of transcription initiation site of human *Flrt3* and at −1008 to −1013 and −2580 to −2586 base pairs upstream of transcription start codon of *Xenopus Flrt3* (Fig. 4A). We first performed chromatin immunoprecipitation (ChIP) analysis to determine whether Tbx2 is recruited to these consensus elements. Of note, Tbx2 associated efficiently with each wild-type T-element sequence in HEK 293 T cells (Fig. 4B). The importance of the T-box binding sites for this association was also examined by site-directed mutagenesis of all two T-element sequences (Fig. 4A). *Flrt3* promoter with mutations at both T-box sites did not associate with Tbx2 (Fig. 4B), indicating that the T-element sequences are necessary for the binding of Tbx2 to *Flrt3* promoter. In addition, we examined the effects of overexpression of *Tbx2* or *BMP4* on the activity of *Flrt3* promoter using luciferase reporter assays. For this, we injected *Flrt3* promoter-driven reporter genes with *Tbx2* or *BMP4* in the dorsal region of embryo where *Tbx2* expression is barely detectable. As shown in Fig. 4C, the activity of *Flrt3* promoter was suppressed by *Tbx2* or *BMP4* in the wild-type reporter construct but not in the mutant construct in which both T-box elements are destroyed. The basal activity of the mutant reporter gene in these assays appears to be lower than that of the wild-type construct. Overall, these results suggest that *Tbx2* down-regulates directly *Flrt3* expression by binding to its promoter.

3.5. *Tbx2* repression of *Flrt3* expression is required for anterior neural development

We next tested whether *Tbx2* repression of *Flrt3* expression is relevant to anteroposterior neural patterning. As shown above, injection of *Tbx2* induced expansion of the expression of anterior neural markers, *Otx2* and *Bf1* (87%, n=42 for *Otx2* and 91%, n=45 for *Bf1*; Fig. 5A-D) but reduction of that of a posterior neural marker, *Krox20*. These effects of overexpressed *Tbx2* were reverted by co-expression of *Flrt3* (65%, n=43 for *Otx2* and 73%, n=42 for *Bf1*; Fig. 5E and F). In addition, overexpression of *Flrt3* inhibited strongly the expression of the anterior neural markers (91%, n=45 for *Otx2* and 93%, n=43 for *Bf1*; Fig. 5G and H). Thus, these results suggest that *Tbx2* inhibition of *Flrt3* expression is crucial for normal anteroposterior neural patterning. Of note, the expression of *Krox20* was also abrogated by overexpressed *Flrt3*, as is the case for *Tbx2*. Given that *Krox20* expression in rhombomeres 3 and 5 in the developing hindbrain depends on FGF signals (Marin and Charnay, 2000; Walshe et al., 2002), and Tbx2 and Flrt3 have opposite effects on FGF signaling (Fig. 3F), it seems likely that the normal expression of *Krox20* requires the fine-tuning of FGF signaling.

Consistent with the ability of Tbx2 to induce the anterior-most structures in a secondary axis assay (Fig. 1), knockdown of *Tbx2* in ectoderm caused malformation of head structures such as the absence of eyes as well as loss of pigments along the antero-posterior body axis (Fig. 6A-C and H). We next assessed, especially focusing on eye formation, whether *Tbx2* suppression of *Flrt3* expression is also relevant to formation of head structures. Notably, the loss of eyes in *Tbx2*-depleted embryos could be rescued by co-injection of *Flrt3* MO but not by CO MO (Fig. 6C-E and H), suggesting that the anteriorizing ability of Tbx2 may result from the repression of *Flrt3* expression which leads to down-regulation of FGF signaling. As FGF signals are involved in both early neural induction and later anteroposterior neural patterning (Dorey and Amaya, 2010), we investigated whether the late inhibition of FGF signaling could also reverse the inhibitory effect of *Tbx2* MO on eye formation. As shown in Fig. 6F-H, treatment with SU5402, a specific inhibitor of FGF signaling, but not DMSO, from stage 15–20 could rescue efficiently the absence of eyes in embryos lacking Tbx2 function. Taken together, these results suggest that *Tbx2* contributes to the anteroposterior patterning of neural tissues and formation of head structures such as eyes by suppressing FGF signaling after the onset of gastrulation.

4. Discussion

The present study has shown that a transcriptional repressor, Tbx2, has an important role in regulation of anteroposterior neural patterning in *Xenopus* embryo. In support of this, the gain-of-function of Tbx2 expands the expression of anterior neural markers but abrogates that of a posterior neural marker. Furthermore, the loss-of-function of Tbx2 gives opposite phenotypes to those caused by its overexpression. During the late gastrula and early neurula stages, Tbx2 is expressed in the anterior region of embryo that appears to include the cement gland and forebrain. This expression pattern resembles the localization at the same stages of phospho-Smad1 immunoreactivity, a downstream indicator of BMP pathway activation (Cho et al., 2013). As Tbx2 expression can be induced by BMP4 signal in the ectoderm (Fig. 2), it might recapitulate as a target gene the activities of BMP signaling pathway in the anterior domain of early embryo. Consistently, inhibition of the late BMP signaling after the mid-gastrulation suppresses the expression of anterior neural markers (Cho et al., 2013), which is similar to the phenotypes caused by Tbx2 knockdown. In addition, a recent study has shown that expression of Tbx2 was eliminated in embryos depleted of subtilisin-like proprotein convertase 7 (SPC7), which is thought to regulate upstream of the BMPs the early patterning of the anterior neural plate and its derivatives (Senturker et al., 2012). Therefore, these findings indicate that Tbx2 functions as a downstream mediator of BMP signaling to specify the anterior forebrain tissue.

Our results suggest that Tbx2 down-regulates FGF signaling to control AP neural patterning. Overexpression of Tbx2 impedes ERK phosphorylation and neural caudalization induced by FGF8 signal. These inhibitory effects appear to be achieved through Tbx2 repression of transcription of *Flrt3*, a positive regulator of FGF signaling. As a result, *Flrt3*-depleted embryos exhibit expanded expression of anterior neural markers but reduced expression of relatively posterior neural markers (Cho et al., 2013), which looks like the phenotypes induced by Tbx2 overexpression. Furthermore, Tbx2 and Flrt3 show antagonistic interaction in control of expression of anterior and posterior neural markers. Like Tbx2, BMP signal has been shown to attenuate FGF signaling in terms of ERK activation and neural posteriorization, and this activity requires transcriptional induction of a negative regulator of the latter pathway (Cho et al., 2013). Notably, BMP signal is not able to inhibit FGF signaling in the absence of Tbx2 function as shown in Fig. 2. Therefore, Tbx2 appears to be such an indirect mediator of BMP signaling to down-regulate FGF pathway. FGF signaling has been shown to attenuate BMP pathway through the MAPK-mediated phosphorylation of Smad1 (Pera et al., 2003; Sapkota et al., 2007). FGF signal can also induce *Flrt3* expression, creating a positive feedback loop (Bottcher et al., 2004). Taking together, we propose a

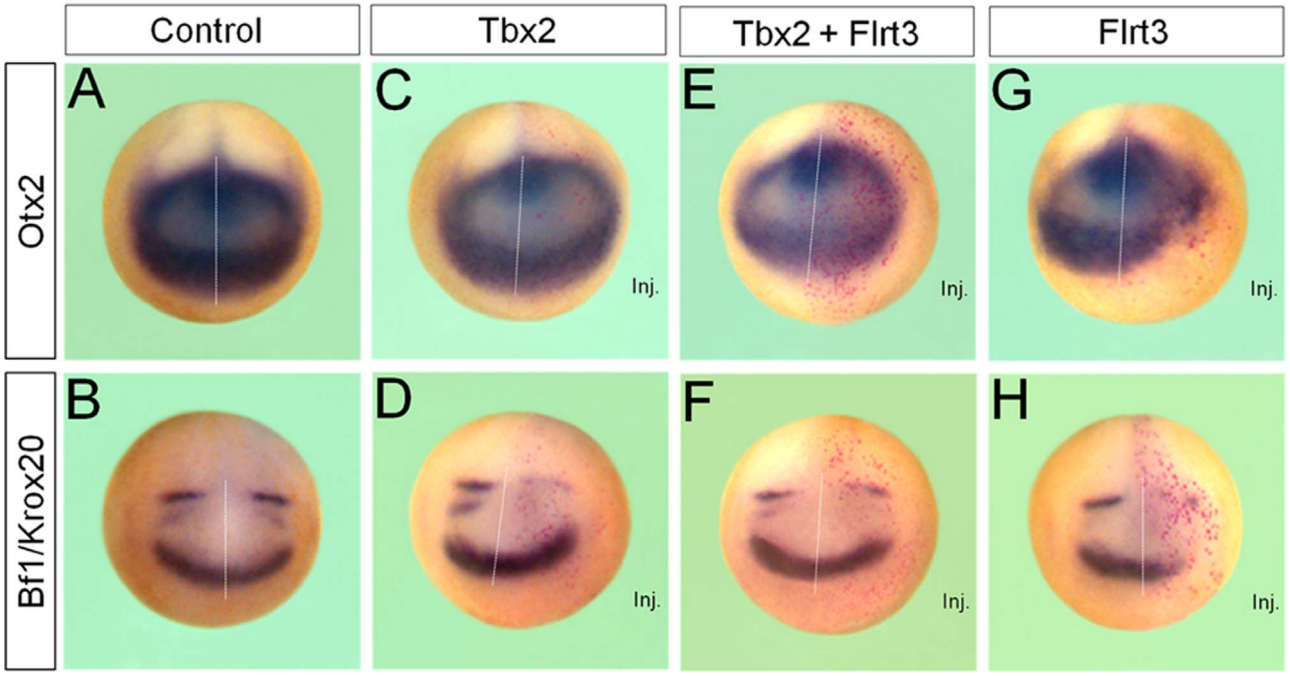


Fig. 5. Tbx2 functions as a repressor of *Flrt3* expression during anterior neural formation. (A-H) One blastomere of four-cell stage embryo was injected dorso-animally as indicated with *Tbx2* (200 pg) and *Flrt3* (200 pg) along with *LacZ* RNA and cultured to stage 15 for *in situ* hybridization against *Otx2*, *Bf1* or *Krox20*. Embryos are shown in anterior view with dorsal to the top. Control, uninjected control embryo. Inj. indicates the injected side of embryo.

model for anteroposterior neural patterning by antagonistic interplay between the BMP/Tbx2 and FGF/Flrt3 pathways as shown in Fig. 7.

On the other hand, BMP4 signaling has been also reported to inhibit MAPK activity *via* a TAK1/p38 pathway without induction of another regulator in *Xenopus* early gastrula ectoderm (Goswami et al., 2001; Liu et al., 2012). Inhibition of the BMP4/TAK1/p38 pathway induces neural fates in naïve ectodermal tissue (Goswami et al., 2001). Given that Ras/MAPK activation is involved in neural induction prior to gastrulation (De Robertis and Kuroda, 2004; Kuroda et al., 2005), the short-term inhibition of FGF/MAPK activity by the BMP4/TAK1 pathway might be critical for promoting epidermal development at the expense of neural cell fates. In contrast, the attenuation of FGF signals by the BMP4/Tbx2 pathway is essential for anterior neural specification after the mid-gastrulation as demonstrated in our study. This long-term control, which involves zygotic induction of a negative regulator such as Tbx2, seems too late to participate in a binary decision between epidermal and neural cell fates through repression of neural inducing FGF signals. The BMP4/TAK1 signal could affect several FGF pathways including FGF1, FGF4, and FGF8, because it inhibits the activity of MAPK, a common downstream effector of these signalings. However, since the target of the BMP4/Tbx2 pathway, Flrt3 shows a preference for FGF8 in modulation of gene expression (Bottcher et al., 2004), this pathway would be specific for FGF8 signaling. Moreover, while FGF8 and Flrt3 are coexpressed in the presumptive mesoderm in gastrulae and in the anterior neural ridge in neurulae (Bottcher et al., 2004; Fletcher et al., 2006), Tbx2 is expressed mainly in the ventral ectoderm and anterior edge of the neural plate during the same stages, suggesting a specific effect of Tbx2 on the neural patterning mediated by the FGF8/Flrt3 signaling. Therefore, the BMP4/Tbx2 pathway appears both spatially and temporally fit for regulating AP neural patterning by attenuating the activity of FGF signal.

As shown in this work, the post-gastrula roles of the BMP4/Tbx2 pathway may include formation of head structure as well as regulation of AP neural patterning. Inhibition of BMP signaling alone in the ventral region of *Xenopus* embryo induces a secondary trunk, whereas combined reduction of BMP and Wnt or Nodal signal induces a complete dorsal axis with an extra head (Glinka et al., 1997; Niehrs, 1999; Piccolo et al., 1999). Of note, *Tbx2* induces strongly a secondary axis with an ectopic head structure when coinjected ventrally with a dominant negative (DN) BMP receptor. In line with the role of Tbx2 in suppressing FGF pathway, *Flrt3* MO or SU5402-mediated inhibition of FGF signaling after the mid-gastrula stages can also rescue the loss of eyes in *Tbx2*-depleted embryos. It has been shown that inhibition of BMP signal derepresses FGF signaling pathway in ectodermal cells (Cho et al., 2013). Likewise, the ventral mesodermal tissue expressing DN BMP receptor would also experience up-regulated FGF signal. Moreover, the level of FGF signal might become much higher in the mesoderm than in the ectoderm, because the continuous supply of FGF ligands such as FGF8 from the mesoderm could induce more expression of *Flrt3*, thereby resulting in a much stronger FGF signal in a positive feedback loop. Thus, it seems likely that formation of anterior-most structures, eyes and cement gland in the secondary body axis might be suppressed by the posteriorizing activity of FGF signal which is derepressed by inhibition of BMP signal. Therefore, the BMP4/Tbx2 pathway may inhibit caudalizing FGF signals, resulting in more efficient formation of anterior head structures.

After anterior neural induction by BMP inhibition in vertebrates, the A-P neural patterning requires not only caudalizing signals such as Wnt and FGF that alter anterior neural to posterior neural tissue but also anti-caudalization signals such as Wnt and FGF antagonists in the anterior region of early embryo. Extracellular Wnt antagonists including Dkk1, Frzb, WIF1, and Del1 (Glinka et al., 1998; Hsieh et al., 1999; Leyns et al., 1997; Takai et al., 2010) are crucial for anterior neural and head formation. Intracellular transcriptional factors such as Tcf3, Six3, and XsalF also contribute to forebrain formation by down-regulating the competence of the anterior neural tissue to respond to posteriorizing Wnt signals (Andoniadou and Martinez-Barbera, 2013; Onai et al., 2004). In addition, Shisa has been shown to act as an inhibitor of both Wnt and FGF signals by interfering with the transport of their receptors from the endoplasmic reticulum to the plasma membrane in the anterior neural plate (Yamamoto et al., 2005). Our data reveals a stage-specific role of the BMP/Tbx2 pathway as a novel anti-caudalization factor which down-regulates FGF signaling for anterior neural development. Future experiments are warranted to investigate whether

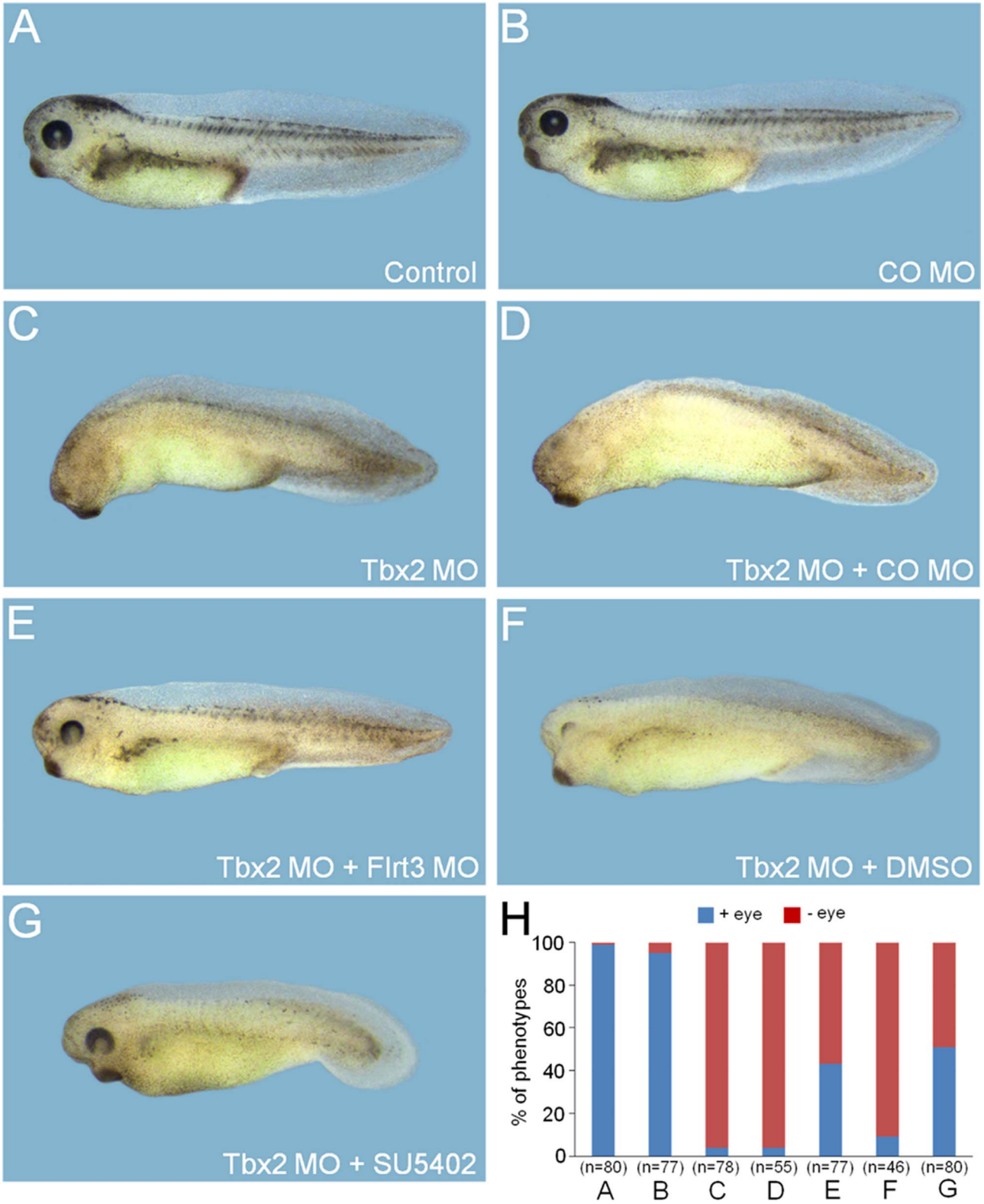


Fig. 6. Tbx2 inhibition of FGF signaling is critical for formation of head structures. (A-G) Four-cell stage embryos were injected in the animal region as indicated with *Tbx2* MO (40 ng), CO MO (60 ng) and *Flrt3* MO (60 ng) and cultured to tadpole stages with or without chemical treatment. SU5402 (20 μM) or DMSO was treated to the injected embryos from stage 15–20. Control, an uninjected embryo. Lateral views with anterior to the left. (H) Quantification of the phenotypes shown in (A-G) in terms of eye formation. n, the total number of embryos analyzed. + eye, embryo with eyes; - eye, embryo with no eyes.

the inhibition of the FGF/Flrt3 pathway by the BMP/Tbx2 signal might occur in developmental events other than neural patterning and to identify other target genes of the BMP/Tbx2 signal which would mediate the crosstalks between BMP and other signaling pathways.

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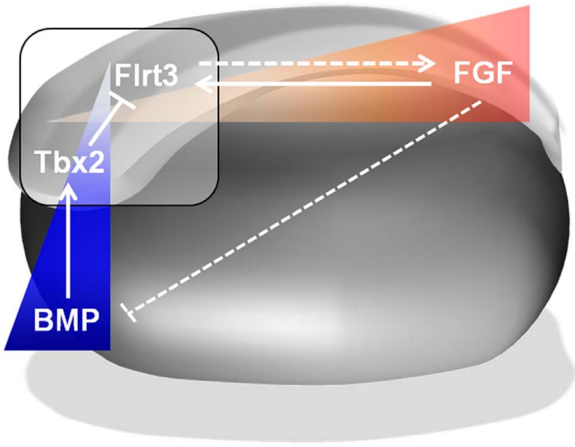


Fig. 7. Model of the proposed mechanism by which Tbx2 inhibits FGF signaling to regulate anterior neural formation. Tbx2 acts as a target gene of BMP signal to suppress the expression of *Flrt3*. Flrt3 potentiates FGF signaling (dashed arrow) and FGF signals can induce *Flrt3* expression, creating a positive feedback loop. On the other hand, FGF signals inhibit BMP signaling by inducing the phosphorylation of Smad1 in its linker region (dashed line). Thus, it appears that the BMP/Tbx2 and FGF/Flrt3 pathways antagonize each other for the correct patterning of anterior neural tissues.

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