BMP inhibition initiates neural induction via FGF signaling and Zic genes

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Neural induction is the process that initiates nervous system development in vertebrates. Two distinct models have been put forward to describe this phenomenon in molecular terms. The default model states that ectoderm cells are fated to become neural in absence of instruction, and do so when bone morphogenetic protein (BMP) signals are abolished. A more recent view implicates a conserved role for FGF signaling that collaborates with BMP inhibition to allow neural fate specification. Using the *Xenopus* embryo, we obtained evidence that may unite the 2 views. We show that a dominant-negative R-Smad, Smad5-somitabun—unlike the other BMP inhibitors used previously—can trigger conversion of *Xenopus* epidermis into neural tissue in vivo. However, it does so only if FGF activity is uncompromised. We report that this activity may be encoded by FGF4, as its expression is activated upon BMP inhibition, and its knockdown suppresses endogenous, as well as ectopic, neural induction by Smad5-somitabun. Supporting the importance of FGF instructive activity, we report the isolation of 2 immediate early neural targets, *zic3* and *foxD5a*. Conversely, we found that *zic1* can be activated by BMP inhibition in the absence of translation. Finally, Zic1 and Zic3 are required together for definitive neural fate acquisition, both in ectopic and endogenous situations. We propose to merge the previous models into a unique one whereby neural induction is controlled by BMP inhibition, which activates directly, and, via FGF instructive activity, early neural regulators such as Zic genes.

xenopus | default model | Smad5-sbn

Neural induction is viewed as a decision made by gastrula ectodermal cells between neural and epidermal fates (1, 2). This process has been best studied in the *Xenopus* and chick embryos, which led to the emergence of distinct molecular models. The default model, based initially on *Xenopus* studies, has proposed that bone morphogenetic protein (BMP) inhibition is necessary and sufficient for neural induction (1). Studies in the chick have implicated additional instructive signals, among which FGF is an early and essential one (3, 4). However, one shared conclusion is that neural fate assignment requires the down-regulation of BMP signals (5, 6). What remains controversial is whether BMP inhibition could be sufficient for neural induction. Recently, we and others have introduced a paradigm to test the validity of the default model in frogs, which consists of micro-injection of cell-autonomously acting BMP inhibitors in ventral ectodermal cells of the 16- or 32-cell embryo (5, 6). Fate mapping combined to marker gene analysis indicate that these blastomeres normally give rise exclusively to epidermal cells (5). Those cells are competent for neuralization, but do not become neural if injected with Smad6 or a dominant-negative BMP receptor (5, 6). However, the epidermal-to-neural switch occurs when a low amount of FGF4 (called eFGF in the frog) is combined with those BMP inhibitors, supporting a combinatorial model (5, 6).

It remained possible, however, that the lack of neuralization of epidermal cells was a result of incomplete BMP inhibition in previous studies. Here, we addressed this possibility by using Smad5-somitabun (Smad5-sbn), an anti-morphic form of Smad5 (one of the 3 BMP pathway R-Smads). This engineered version of murine Smad5 contains a mutation in the L3 loop, at the same position as the *somitabun* mutation in the zebrafish orthologue (7). This mutation is thought to prevent binding of Smad5 to the co-Smad Smad4, but not to Smad5 itself. As the fish *somitabun* mutant can be rescued by Smad5, as well as Smad1 (7), it suggests that Smad5-sbn could form inactive heteromeric complexes with Smad5, Smad1, and perhaps also Smad8, thus efficiently shutting down BMP signaling at the lowest integration point in the pathway. We found that Smad5-sbn could robustly induce neural tissue in epidermis, but only if FGF4 activity was maintained.

Understanding the roles of FGF signaling and BMP inhibition in neural induction requires the identification of their transcriptional targets. Here, we focused our attention on the early neural genes *zic1*, *zic3* and *foxD5a*. The zinc finger-containing transcription factors Zic1 and Zic3 are expressed in the dorsal ectoderm before gastrulation, encompassing the domain of *sox2* expression, which is regarded as the first definitive neural marker (8, 9). We found that *zic3* and *foxD5a*, but not *zic1*, are activated by FGF in absence of translation, suggesting that they are direct targets of this pathway. Conversely, *zic1*—but not *zic3* and *foxD5a*—is an immediate early target of BMP inhibition. This last result is consistent with the existence in the *zic1* promoter of a module, called the BMP inhibition responding module, that is sufficient for expression in response to BMP inhibition, although the mechanism of activation appears to be complex (10). It was reported that both Zic1 and Zic3 promote neural and neural crest fates when overexpressed in animal cells (8, 9). However, their actual role in neural induction has never been addressed by loss-of-function analyses to our knowledge. By using antisense morpholino-mediated knockdown, we show that Zic1 and Zic3 together are required for the progression through the neural program.

This work definitively validates the default model of neural induction in the most relevant and selective in vivo assay. It also reconciles this model with the proposed instructive role of FGF signaling, which is necessary downstream of BMP inhibition to initiate the neural program.

Results

In Vivo Neural Induction by BMP Inhibition. In an effort to look for means of inducing neural tissue by BMP inhibition, we tested Smad5-sbn in our in vivo neural induction assay (5). Unexpectedly, when *smad5-sbn* mRNA was injected in the ventral-most animal blastomeres of 16-cell embryos (here designated AB4 cells), a robust and reproducible activation of the neural markers *sox2* and *sox3*, accompanied by the loss of the epidermal marker *k81*, was visible at the late gastrula stage

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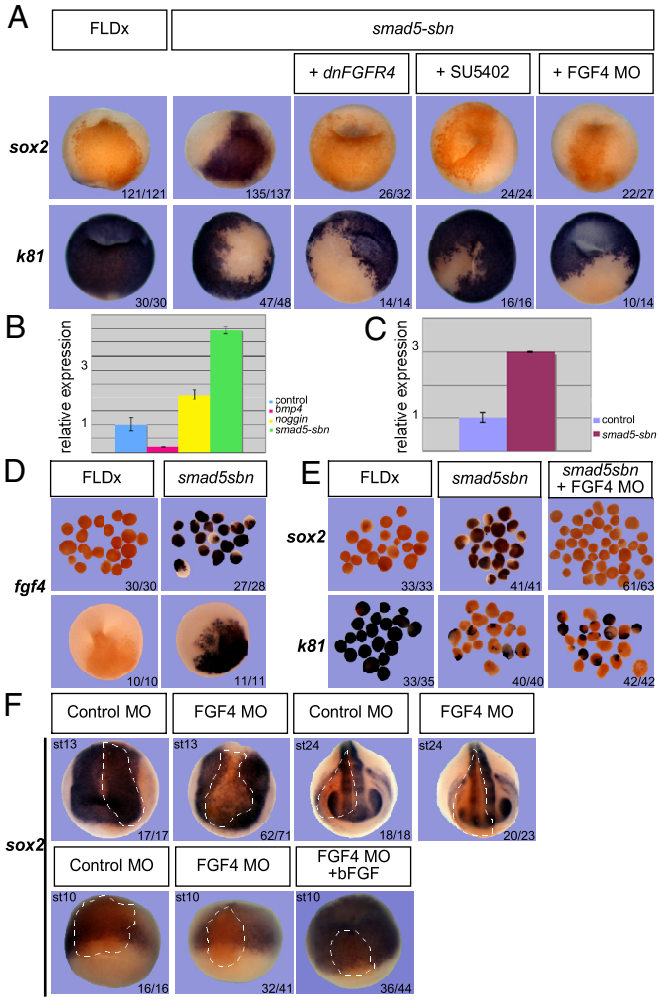

Fig. 1. In vivo neural induction by Smad5-sbn requires FGF activity. (*A*) Ventral views of stage 13 embryos injected at 16-cell stage in one AB4 blastomere with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA, 1 ng *dnFGFR4* mRNA, 23 ng *FGF4* MO, or treated with 200 μM SU5402, as indicated. In this and the following figures, the orange staining reveals the presence of the lineage tracer FLDx. (*B*) Quantitative RT-PCR analysis of *FGF4* expression at stage 10.5 of embryos injected in all cells at 4-cell stage with 1 ng/blastomere *bmp4* mRNA, 1 ng/blastomere *noggin* mRNA, or 3 ng/blastomere *smad5-sbn* mRNA. For this and all following quantitative RT-PCR graphs, expression levels were normalized to levels of ornithine decarboxylase (ODC). (*C*) Quantitative RT-PCR analysis of *FGF4* expression at stage 10 of animal caps taken at late blastula stage from embryos injected animally in all cells at 4-cell stage with FLDx alone or with 3 ng/blastomere *smad5-sbn* mRNA. (*D*) *Top*: Animal caps as in *C*. *Bottom*: Ventral views of stage 10 embryos injected with FLDx alone or with 3 ng *smad5-sbn* mRNA in AB4 at 16-cell stage. (*E*) Stage 13 animal caps taken at late blastula stage from embryos injected as in *C*, in the presence or absence of 23 ng/blastomere *FGF4* MO. (*F*) Dorsal views of stage 10, stage 13, and stage 24 embryos injected marginally with 46 ng control MO or 46 ng FGF4 MO in one dorsal cell at 4-cell stage. The injected region is circled with a white dotted line. For the rescue assay, 350 pg of recombinant bFGF protein was injected in the blastocele at stage 8. In this and the following figures, the number of embryos or caps exemplified by the photograph over the total number analyzed is displayed.

[Fig. 1*A* and supporting information (SI) Fig. S1*A*]. Lineage tracing with a fluorescent dextran revealed that neural gene activation occurred only in the injected domain, suggesting that Smad5-sbn acted primarily in a cell-autonomous manner (Fig. 1*A* and Fig. S1*A*). We found that both *sox2* and *sox3* were activated by Smad5-sbn between gastrula stages 10 and 10.5 (Fig. S1*A*). The induced neural tissue expressed *otx2*, but not *hoxA7*, suggesting that it is of anterior character (Fig. S1*B*). Finally, the neuralization by Smad5-sbn was stable, as revealed by the expression of the late marker neural cell adhesion molecule (NCAM) at tailbud stages (Fig. S1*C*). We conclude that induction by Smad5-sbn recapitulates normal neural induction. It was recently reported that combined Smad1 and Smad2 inhibition could induce neural tissue in epidermal precursors in vivo (11). Therefore, we tested whether Smad5-sbn could also antagonize Smad2. We injected *smad5-sbn* into the dorsal marginal zone of 4-cell embryos and analyzed markers known to depend on Nodal/Smad2 signaling. We found that Smad5-sbn did not repress the mesoderm marker *Xbra*, and actually caused an expansion of *chordin* and *goosecoid*, as expected from the negative effect of BMP signaling on organizer genes (Fig. S2*A*). Moreover, those markers were not activated upon *smad5-sbn* injection in AB4 cells, suggesting that neural induction in this assay is not a response to the production of dorsal mesoderm (Fig. S2*B*). To verify that Smad5-sbn functions via BMP R-Smad inhibition, we co-injected it with LM-Smad1, a mutant that retains the ability to transduce BMP signals, but resists inhibition by ERK (12). As expected, LM-Smad1 suppressed the neural tissue induced by Smad5-sbn, and restored epidermis (Fig. S2*C*). We conclude that Smad5-sbn constitutes the first cell-autonomously acting BMP inhibitor able to convert epidermis into neural tissue at distance from the endogenous neural plate.

FGF4 Is Required for Both Normal and Ectopic Neural Induction. Using anti-morphic reagents, we addressed the requirement for FGF activity in Smad5-sbn-induced neural tissue. We found that neuralization of AB4 cells by Smad5-sbn was antagonized by the co-injection of the dominant-negative FGFR4 receptor, and by SU5402 (a pharmacological inhibitor of FGF receptors) treatment (Fig. 1*A*). Unlike the combination of Smad5-sbn and LM-Smad1, the repression of *k81* caused by Smad5-sbn was unchanged in absence of FGF activity, indicating that BMP inhibition was still elevated (Fig. 1*A*). Thus, neural induction by BMP inhibition depends on the presence of FGF activity. This activity could be resident in the ventral ectoderm or induced by BMP inhibition. The second possibility is supported by the previously reported activation of ERK by BMP inhibitors (13). To gain insight into this issue, we tested whether BMP inhibition could activate *FGF4* expression, as this ligand efficiently complements Smad6 in our ectopic neural induction assay (5). Quantitative RT-PCR revealed that *FGF4* expression in whole embryos was activated by injection in all cells of *smad5-sbn*, and *noggin* mRNAs, whereas it was repressed by *bmp4* (Fig. 1*B*). Moreover, Smad5-sbn activated *FGF4* expression in AB4 descendants and in animal caps (Fig. 1 *C* and *D*). Thus, neural induction by BMP inhibition might depend on induced *FGF4* activity. We addressed this possibility by knocking down FGF4 with a translation-blocking morpholino-modified antisense oligonucleotide (MO) (14). We found that FGF4 knockdown suppressed *sox2* activation by Smad5-sbn in AB4 cells, as well as in animal caps, without restoring *k81* expression (Fig. 1*A* and *E*). Moreover, endogenous *sox2* expression in the developing neural plate was down-regulated upon *FGF4* MO injection in the marginal zone, the normal site of expression of *FGF4* (Fig. 1*F*). This repression was visible from the early gastrula to tailbud stage (Fig. 1*F*). Importantly, *sox2* expression was recovered in FGF4 morphant embryos injected at blastula stage with recombinant bFGF protein in the blastocele, confirming that the lack of neural induction could be attributed to decreased FGF activity (Fig. 1*F*). At the dose used in this assay, *Xbra* was not activated by bFGF, suggesting that the rescue did not require the presence of mesoderm. These data suggest that FGF4 is important for neural ectoderm development, although its expression is not detectable in this tissue (15). This could be explained by the

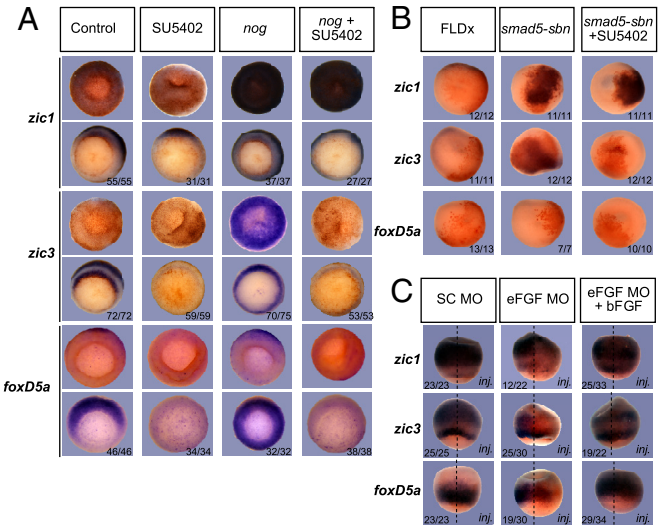

Fig. 2. *zic1*, *zic3*, and *foxD5a* show differential regulation by BMP and FGF signals. (*A*) SU5402 treatment (200 μM) was from 4-cell stage to stage 10.5, and *noggin* mRNA (1 ng/blastomere) was injected in all cells at 4-cell stage. *zic3* and *foxD5a*, but not *zic1*, activation by Noggin depends on FGF activity. For each marker, animal (*Top*) and vegetal views (*Bottom*) are shown. (*B*) Ventral views of stage 13 embryos injected in one AB4 blastomere with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA, or treated with 200 μM SU5402. (*C*) Dorsal views of stage 10.5 embryos injected marginally with 46 ng control MO or 46 ng FGF4 MO in one of the 2 dorsal cells at 4-cell stage. For the rescue assay, 350 pg of bFGF protein was injected in the blastocele at stage 8. Dotted line represents midline; *inj*, injected side.

loss of organizer-specific neural inducers in FGF4 morphants, and/or the capacity of the FGF4 ligand to travel and signal from the dorsal mesoderm to the overlying dorsal ectoderm. To address the first possibility, we analyzed the expression of the genes encoding the BMP antagonists *chordin*, *noggin*, and *cerberus* and the neuralizing factor *FGF8* in FGF4 morphant embryos. We found no visible alteration in *FGF4*-MO-injected cells of *chordin*, *noggin*, and *cerberus* expression, whereas *FGF8* was slightly down-regulated and *Xbra* was repressed as previously reported (Fig. S3) (14). Altogether, our data suggest that FGF signaling is required for neural induction independently of BMP inhibition.

The Neural Genes *zic1*, *zic3*, and *foxD5a* Are Differentially Regulated by BMP and FGF Signals. The current and previous evidence suggest that BMP inhibition and FGF signaling may control distinct effector genes required for deployment of the neural program. We thus looked for candidate FGF and BMP target genes among the earliest known neural regulators. For this, we analyzed the expression of a large number of such candidates in embryos treated with SU5402 or injected with *noggin* mRNA. We selected for further analysis 3 genes, *zic1*, *zic3*, and *foxD5a*, as their expression was activated by Noggin and repressed by FGF inhibition (Fig. 2*A*). Thus, upon radial injection of *noggin* mRNA, both *zic1* and *zic3* were ectopically activated in the whole animal region (Fig. 2*A*). Interestingly, whereas *zic1* animal expression was maintained in *noggin*-injected embryos subjected to SU5402 treatment, *zic3* expression was totally suppressed (Fig. 2*A*), suggesting that these close relatives are differentially regulated by FGF and BMP signals. Concerning *foxD5a*, we found that *noggin* injection led to its radial expression in the marginal ectoderm, but not in more animal domains (Fig. 2*A*). This equatorial activation was lost in the presence of SU5402, indicating that BMP inhibition is not sufficient for *foxD5a* expression (Fig. 2*A*). We then used AB4 injection of Smad5-sbn as a second paradigm to address the response of these 3 genes to BMP and FGF inhibition. In agreement with the effect of Noggin injection, we found that *zic1* and *zic3*, but not *foxD5a*, could be activated by Smad5-sbn in ventral epidermis (Fig. 2*B*). When SU5402 was applied to Smad5-sbn-injected embryos, *zic1*, but not *zic3*, expression was maintained (Fig. 2*B*). Finally, we confirmed that FGF4 plays an important role in the control of *zic1*, *zic3*, and *foxD5a*. FGF4 morphant embryos exhibited a severe down-regulation of all 3 genes that could be rescued by bFGF blastocelic injection (Fig. 2*C*).

zic1 and *zic3* are first expressed at late blastula stages and constitute some of the earliest known neural markers. Therefore, we addressed whether FGF activity and BMP inhibition are involved in this early phase of activation. We found that *zic1* expression was not initiated at the late blastula stage 9 in embryos injected with recombinant BMP4 protein in the blastocele, but was normally activated in the presence of SU5402 (Fig. S4*A*). Conversely, the initiation of *zic3* expression at stage 9 did not take place in SU5402-treated embryos, whereas it was not affected by the presence of excess BMP4 protein (Fig. S4*A*). Both genes were repressed by either treatment by the onset of gastrulation, which can be explained by the mutual repression of FGF and BMP signals, uncovered in this and previous studies (5, 12, 13). Collectively, these data indicate that FGF signaling and BMP inhibition are responsible for the initiation of *zic1* and *zic3* expression, respectively.

***zic3* and *foxD5a* Are Immediate Early Targets of FGF Signaling.** We then wanted to test whether FGF signaling was able to directly activate the expression of these genes. We set out to address this question in animal caps, via the use of recombinant bFGF protein, in the presence or absence of cycloheximide (CHX), a translation inhibitor. In this type of assay, CHX prevents the accumulation of putative intermediate activators, so induced expression likely reflects direct transcriptional activation, although the contribution of post-transcriptional regulatory events mediated by micro-RNAs cannot be ruled out. Unfortunately, CHX alone activated all 3 genes in animal caps, thus preventing us from using this procedure (Fig. S4*B*). Transcriptional activation by CHX in animal caps has been observed for other genes, including *xnr4* and *goosecoid*, and may involve removal of transcriptional repressors (16). As blastocelic injection of bFGF could ectopically activate *sox2* in animal cells (Fig. 1*F*), we reasoned that it could offer an alternative paradigm to answer our question. In whole embryos, CHX treatment alone did not activate any of the 3 genes (Fig. 3*A*). We thus went on to inject various amounts of recombinant bFGF protein in the blastocele of gastrulating embryos. At the highest dose tested (2 ng), all 3 genes were activated, whereas only *zic3* expression was induced at the lowest dose (0.02 ng; Fig. 3*A*). When applied to bFGF-injected embryos, we found that CHX did not prevent *zic3* and *foxD5a* activation. In contrast, *zic1* activation was lost, confirming the efficiency of the CHX treatment and indicating that this gene is indirectly activated by high amounts of bFGF (Fig. 3*A*). As FGF may act via ERK to phosphorylate and inhibit Smad1, it could interfere with BMP signaling in the absence of translation. Thus, BMP inhibition could contribute to neural gene activation by FGF. To evaluate this possibility, we examined *zic3* and *foxD5a* expression following injection in AB4 of *FGF4* in the presence or absence of *LM-Smad1*. Both genes could be induced at blastula stage by FGF4, irrespective of the presence of LM-Smad1 (Fig. S4*C*). We conclude that FGF4 can activate *zic3* and *foxD5a*, despite the presence of BMP activity. Interestingly, the low dose of bFGF activated *zic3* but not *foxD5a* in a CHX-resistant manner (Fig. 3A). In normal embryos, *foxD5a* is restricted to the marginal ectoderm, whereas *zic3* is also present more animally (Fig. 3*A*). As ERK activation is known to be higher in marginal than in animal regions (17), we propose that

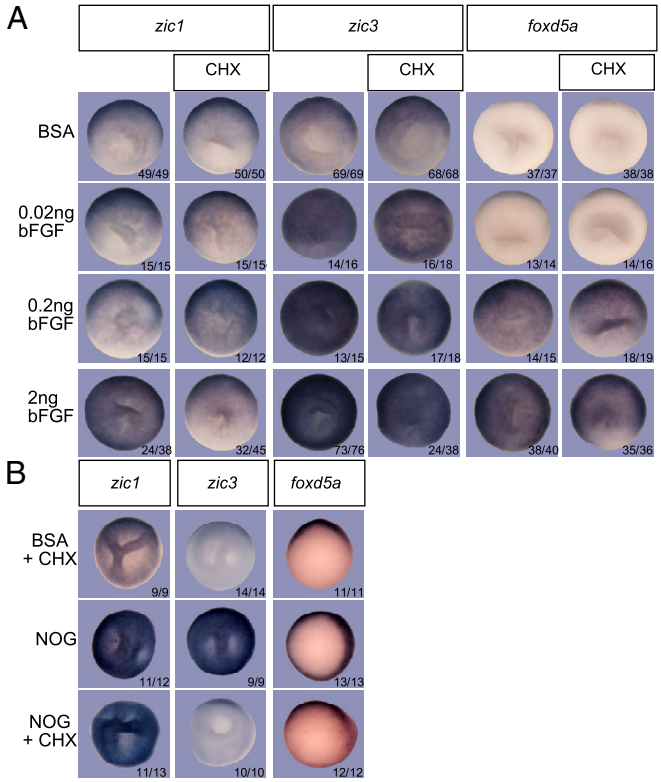

Fig. 3. *zic3* and *foxD5a* are directs targets of FGF signaling, whereas *zic1* is a direct target of BMP inhibition. (*A*) Animal views of stage 11.5 embryos injected at stage 10.5 in the blastocele with 40 ng BSA alone or combined with increasing amounts of bFGF protein, and treated with 10 μg/mL CHX or untreated. (*B*) Stage 8.5 embryos were injected in the blastocele with 40 ng BSA alone or combined with 36 ng Noggin protein in the presence or absence of CHX, and analyzed at stage 10. Animal views are shown for *zic1* and *zic3*, vegetal views for *foxD5a*.

FGF signaling could function as a morphogen in the blastula/gastrula ectoderm.

In conclusion, we identified *zic3* and *foxD5a* as the first CHX-resistant neural targets of FGF signaling in *Xenopus*, providing strong support to the idea that this pathway plays an essential BMP-independent role.

***zic1* Is an Immediate Early Target of BMP Inhibition.** We showed that *zic1* expression can be activated by Noggin in an FGF-independent manner, raising the possibility that this gene is a direct target of BMP inhibition. We tested this idea in embryos injected with Noggin protein in the blastocele and treated with CHX or untreated. As expected, Noggin injection led to pan-animal activation of *zic1* and *zic3* and radial marginal expression of *foxD5a* (Fig. 3*B*). Noggin-dependent activation of *zic3* and *foxD5a* was clearly suppressed by CHX, indicating that these 2 genes are not directly activated by BMP inhibition (Fig. 3*B*). In contrast, the activation of *zic1* by Noggin persisted in the presence of CHX (Fig. 3*B*), suggesting that *zic1* may be a direct transcriptional target of BMP inhibition, in agreement with the presence of a BMP inhibition-responding module in its promoter (10).

***zic1* and *zic3* Are Required for Progression of the Neural Program.** We next asked whether the neural targets identified in our search are functionally important for neural induction. We focused on the *zic* genes, as *foxD5a* was not activated by Smad5-sbn, suggesting that it is dispensable for *sox2* initiation. We used MOs targeting the translation start sites of *zic1* (Zic1 MO) (18), and *zic3* (Zic3 MO1), as well as a second MO designed to block splicing of the first intron in *zic3* (Zic3 MO2). We verified by RT-PCR that Zic3 MO2 indeed provoked exon 2 skipping, which is predicted to yield a truncated inactive protein (Fig. S5). We analyzed the effects of our MOs used alone or in combination and in endogenous or Smad5-sbn-induced neural tissue. We found that neither Zic1 MO, nor Zic3 MO1 or MO2 injected alone, could totally suppress *sox2* expression induced by Smad5-sbn (Fig. 4 *C–E*). Upon co-injection, however, Zic1 MO and Zic3 MO1 efficiently suppressed induction of *sox2* expression by Smad5-sbn (Fig. 4*F*). The combination of Zic1 MO and Zic3 MO2 also antagonized Smad5-sbn (Fig. 4*G*). A similar outcome was obtained upon injection of Zic MOs in the prospective neural plate. Each MO injected alone only weakly affected the endogenous *sox2* expression, whereas the combination significantly decreased or suppressed it at early and late stages of development (Fig. 4 *I–L*, *N–P*, *R*, and *S*). We obtained several lines of evidence supporting the specificity of action of the MOs used here. We found that Zic3 MO2 behaved identically to Zic3 MO1, as it provoked severe *sox2* repression when combined with the published Zic1 MO (Fig. 4*L*), but not by itself (Fig. 4*J*) or combined with Zic3 MO1 (Fig. 4*K*). More importantly, the lack of *sox2* expression in Zic1/Zic3 morphants could be efficiently rescued at all stages by the presence of *zic1* or *zic3* mRNAs, which do not display significant overlap with any of our MOs (Fig. 4 *M* and *Q*). Several important conclusions can be drawn when analyzing double knocked-down embryos. First, as *sox2* expression was lost at the early gastrula stage, it supports the idea that the Zic proteins are required to initiate the neural program (Fig. 4*R*). Second, the neural deficiency is irreversible, as *sox2* expression was absent in morphant embryos at tailbud stage (Fig. 4*S*). Last, both anterior and posterior neural markers are suppressed, consistent with a role of Zic1 and Zic3 in global neural induction (Fig. 4 *T–W*). Among all conditions tested, only those including Zic1 and either of the Zic3 MOs provoked robust and highly penetrant neural deficiencies. The lack of individual phenotypes could be a result of partial inhibition by each MO, or could reflect the existence of a compensatory mechanism between the 2 *zic* genes. We addressed the second possibility. By using quantitative RT-PCR, we found that *zic1* expression was up-regulated upon Zic3 knockdown (twofold), and that *zic3* expression was up-regulated upon Zic1 knockdown (fourfold; Fig. 4*X*). *Sox2* expression was not significantly modified in either case, whereas it collapsed nearly totally upon double knockdown (Fig. 4*X*). Thus, it is likely that the overall amount of Zic activity is unchanged upon single knockdown as a result of up-regulation of the non-targeted *zic* gene. Together, these data indicate that Zic1 and Zic3 act redundantly to promote the neural program.

Discussion

In its initial wording, the default model stated that BMP inhibition was necessary and sufficient for neural induction (1). Our data with Smad5-sbn confirm that this assertion was correct. However, neural induction by Smad5-sbn involves the activation of FGF4, indicating that BMP inhibition and FGF signaling are both important, and act sequentially. Several lines of evidence support the BMP-independent role of FGF signaling in neural induction. First, we can rule out a mode of action limited to BMP inhibition via ERK-mediated Smad1 phosphorylation (12). As Smad5-sbn acts by depleting the pool of endogenous R-Smads available to transduce BMP signals, it functions downstream of ERK, and thus should be insensitive to FGFR inhibition. Consistent with this idea, we showed that FGF4 can activate its targets in the presence of LM-Smad1, a mutant resistant to inhibition by ERK. Second, we obtained evidence that BMP inhibition is still active when FGF signaling is antagonized in Smad5-sbn embryos. The epidermal program, marked by *k81* expression, is not recovered, whereas the target gene *zic1* is still expressed in such embryos. Third, the BMP antagonists *chordin*,

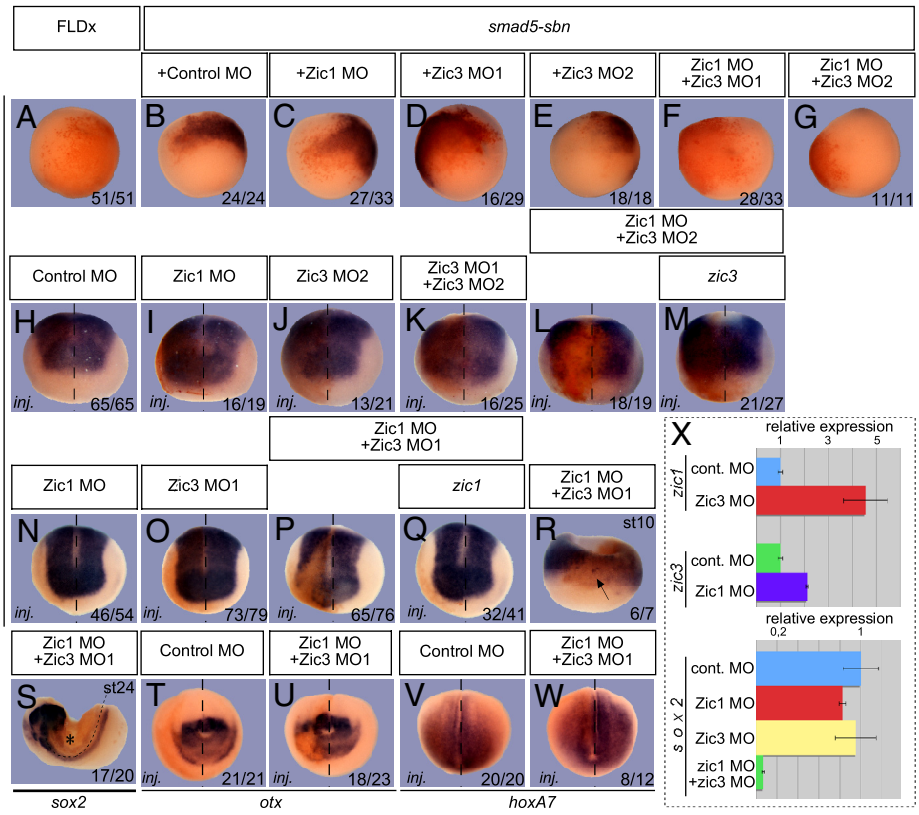

Fig. 4. Zic1 and Zic3 together are required for neural fate specification. (*A–G*) Ventral views of stage 13 embryos injected in one AB4 blastomere at 16-cell stage with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA, and 23 ng control MO, 23 ng Zic1 MO, 23 ng Zic3 MO1, 23 ng Zic3 MO2, a mixture of 23 ng Zic1 MO and 23 ng Zic3 MO1, or a mixture of 23 ng Zic1 MO and 23 ng Zic3 MO2, as indicated. (*H–W*) Eight-cell embryos were injected in the 2 right animal blastomeres with 2.5 ng/blastomere FLDx and the indicated MOs (same amounts listed earlier). The combined knockdown of Zic1 and Zic3 leads to the suppression of *sox2* expression at stage 10 (arrow in *R*), at stage 13 (*L* and *P*), and at stage 23 (*S*). Note the lack of *sox2* staining in the posterior brain and spinal chord (asterisk in *S*). For rescue assays, 4-cell embryos were injected in the 2 right blastomeres with 500 pg *zic3* (*M*) or *zic1* (*Q*) mRNAs before MO injection. (*H–Q*) Dorso-anterior views at stage 13; (*R* and *S*) dorsal views; (*T* and *U*) anterior views at stage 15; and (*V* and *W*) posterior views at stage 15. (*X*) Four-cell embryos were injected with control or Zic MOs (same amounts as listed earlier in each of the 4 cells) and collected for quantitative RT-PCR analysis at stage 10.5.

noggin, and *cerberus* are still expressed in FGF4 morphants that lack neural gene expression. Finally, we showed that FGF signaling can activate *zic3* and *foxD5a* in a translation-independent manner, whereas BMP inhibition cannot.

Why is Smad5-sbn capable of neuralizing the epidermis, whereas Smad6 or the dominant-negative BMP receptor are not? We could think of 2 possibilities: that Smad5-sbn does not only behave as a BMP inhibitor, or that Smad5-sbn inhibits BMP signaling more potently than the other anti-morphic reagents. The evidence presented here does not support the first possibility. First, Smad5-sbn induces neural tissue in the absence of dorsal mesoderm markers, making indirect neural induction in our paradigm highly unlikely. Second, the lack of repression of *chordin* and *goosecoid* expression by Smad5-sbn indicates that it does not inhibit Wnt and Nodal signaling, which are known to regulate these genes and limit neural specification (11, 19, 20). Therefore, in absence of current contradictory evidence, we suggest that Smad5-sbn acts as a specific and powerful BMP inhibitor.

Our work illustrates how FGF signaling and BMP inhibition may directly translate into transcriptional responses in prospective neural territories. We found that, in the absence of translation, *zic3* and *foxD5a* are activated by FGF signals, whereas *zic1* is activated by BMP inhibition. *zic1* and *zic3* both mark the entire presumptive neural plate at the early gastrula stage, and become progressively restricted to the neural/non-neural border as gastrulation proceeds (8, 9). Thus, these 2 genes are not definitive neural markers. They are nonetheless functionally required for the proper expression of the definitive neural marker *sox2* in the neural plate, or in neural tissue induced by Smad5-sbn. Interestingly, we uncovered a compensatory mechanism between the 2 genes that may ensure sufficient Zic activity when one member is inactivated, further highlighting their essential role. Functional redundancy has also been observed between *zic* genes in mutant mice, including in the *zic1*/*zic3* double-knockout animals (21). Future work should address whether the role of Zic factors in neural induction is evolutionary conserved. Members of this family are required in ascidian embryos for neural fate emergence, suggesting that this function might indeed be ancestral (22, 23).

Our data help to reveal the sequence of events that control the engagement of ectoderm cells into the neural program. At the top of this sequence, BMP inhibition, acting partly via FGF4, biases the choice of pluripotent ectoderm cells toward a neural identity, marked by *zic* gene expression. In agreement with this view, the initiation of *zic1* and *zic3* expression at the late blastula stage is prevented in embryos subjected to BMP4 protein or SU5402, respectively. Moreover, we found that neural induction efficiently occurs in embryos exposed to excess Noggin protein before, but not after, the onset of gastrulation (Fig. S6*A*). Following this inductive reaction, the Zic transcription factors contribute to initiate the expression of *sox2*, thus committing early gastrula ectodermal cells to a neural identity. This regulatory cascade is illustrated by the sequential activation from mid-blastula transition to early gastrula, of *FGF4*, *zic3*, *zic1*, and *sox2* in epidermal progenitors subjected to Smad5-sbn (Fig. S7).

However, we would like to argue that the definitive engagement of *zic*/*sox2*-positive cells in the neural program requires a phase of maintenance. This is based on the observations that *sox2* expression in the developing neural plate can be suppressed by exposure to BMP4 protein or ectopic Smad2 activity until mid-gastrulation (11) (Fig. S6*B*).

Work in the chick revealed a similar sequence, as FGF signaling is known to activate the expression of "pre-neural, pre-forebrain" markers, that are all expressed before the definitive neural marker *sox2* (2). There is, however, a critical difference between data in the chick and our data, as BMP inhibition appears unable to initiate neural induction in competent chick epiblast (2). Based on our findings, however, it remains possible that this difference could be attributed to insufficient BMP inhibition in former chick studies. Smad5-sbn may offer the perfect tool to further address this critical issue.

Materials and Methods

Embryo Manipulations and Injections. Eggs obtained from NASCO females were fertilized in vitro, de-jellied, cultured, staged, and injected as described (5, 24). Synthetic capped mRNAs were produced with Ambion mMessage mMachine kit (see *SI Materials and Methods* for references of expression constructs). Previously described MOs were used according to the original references: FGF4 (14) and Zic1 (18). Zic3 MOs were obtained from GeneTools LLC. Sequences were as follows: Zic3 MO1, 5′-TCCTCCATCTAATAGCATTGTCATG-3′; Zic3 MO2, 5′-CTTCTCACCTGGAAAAATATGCAGA-3′. As we noticed interference of MOs with RNAs in solution, we performed separate injections. Fixable fluorescent lysine dextran (FLDx; 2.5 ng/cell) was co-injected with MOs as a lineage tracer. All injections were performed twice or more to establish reproducibility.

Chemical and Protein Treatments. SU5402 (Calbiochem) was dissolved in DMSO (120 mM) and diluted in 0.1× modified Barth solution for whole-embryo treatments. Recombinant human bFGF (2 ng/embryo; Sigma), recombinant human Noggin (36 ng/embryo; R&D Systems), and recombinant human BMP4 (R&D Systems; 2 ng/embryo) proteins were resuspended as recommended by the provider and injected in the blastocele of embryos at blastula or gastrula stages. CHX treatment (10 μg/mL) was started 45 min before bFGF or Noggin protein injection to avoid any delay of action, and treatment was continued for 2.5 h at 18 °C.

In Situ Hybridization, Immunostaining, and Quantitative RT-PCR. Injected embryos were processed for whole-mount in situ hybridization (WISH) with digoxigenin-labeled probes (Roche) as described (25), with some modifications (see SI Materials and Methods). Following staining with BM Purple (Roche), pigmented embryos were bleached, and FLDx was detected by incubation with an anti-fluorescein/alkaline phosphatase antibody (dilution 1/10,000; Roche) and staining with iodonitrotetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche) (6). For quantitative RT-PCR, we use the following primer pairs: *zic1* forward primer, 5′ gcc aat agc agt gat cgt aaa 3′; *zic1* reverse primer, 5′ ttg gga aga tgc ttc gtg 3′; *zic3* forward primer, 5′ tat cag ggt gca tac cgg aga 3′; and *zic3* reverse primer, 5′ gca aac ctt cta tcg cag cc 3′ (see SI Materials and Methods for previously reported primers). For both *zic1* and *zic3* PCR, annealing was at 55 °C for 15 s and elongation at 72 °C for 45 s. Total RNAs were extracted with the RNeasy mini kit (Qiagen), cDNAs were synthesized using the SuperScript II reverse transcriptase (Invitrogen), and amplifications were performed in the presence of SYBR Green mix (Invitrogen) on an iQ5 machine (Bio-Rad).

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