Supporting Information

Marchal et al. 10.1073/pnas.0906352106

SI Materials and Methods

Expression Constructs. Initial references for our expression constructs are as follows: *dnFGFR4* (1), *noggin* (2), *bmp4* (3), *smad5-sbn* (4), *zic1* (5), *zic3* (6), *FGF4* (7), and *LM-Smad1* (8).

In Situ Hybridization and Probes and Immunostaining. The following modifications were introduced to the protocol by Gawantka and colleagues (9): (*i*) methanol was replaced by ethanol; (*ii*) post-fixation following proteinase K treatment was omitted; (*iii*) hybridization and washes were performed at 60 °C; and (*iv*) incubation with the anti-digoxigenin antibody was done in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) and washes were done in MAB supplemented with 0.1% Triton X-100. Antisense riboprobes for *sox2* (5), *sox3* (10), *cerberus* (11), *fgf8* (12), *chordin* (13), *noggin* (2), *xbra* (14), *goosecoid* (15), *otx2* (16), *hoxA7* (17), *zic1* (18), *k81* (19), *zic3* (6), and *foxD5a* (20) were prepared as described in the respective references. The *FGF4* probe was prepared from a clone in pCS2+ (gift from Harv Isaacs, York, United Kingdom). The plasmid was linearized with HindIII and transcribed with T7. Anti-NCAM immunostaining was carried out with the 4d antibody as described (20).

Quantitative RT-PCR. The following primers were used as described in the original references: *fgf4* reported by Kofron et al. (22), *sox2* reported by Mir et al. (23), and ODC used as an internal reference as reported by Heasman et al. (24).

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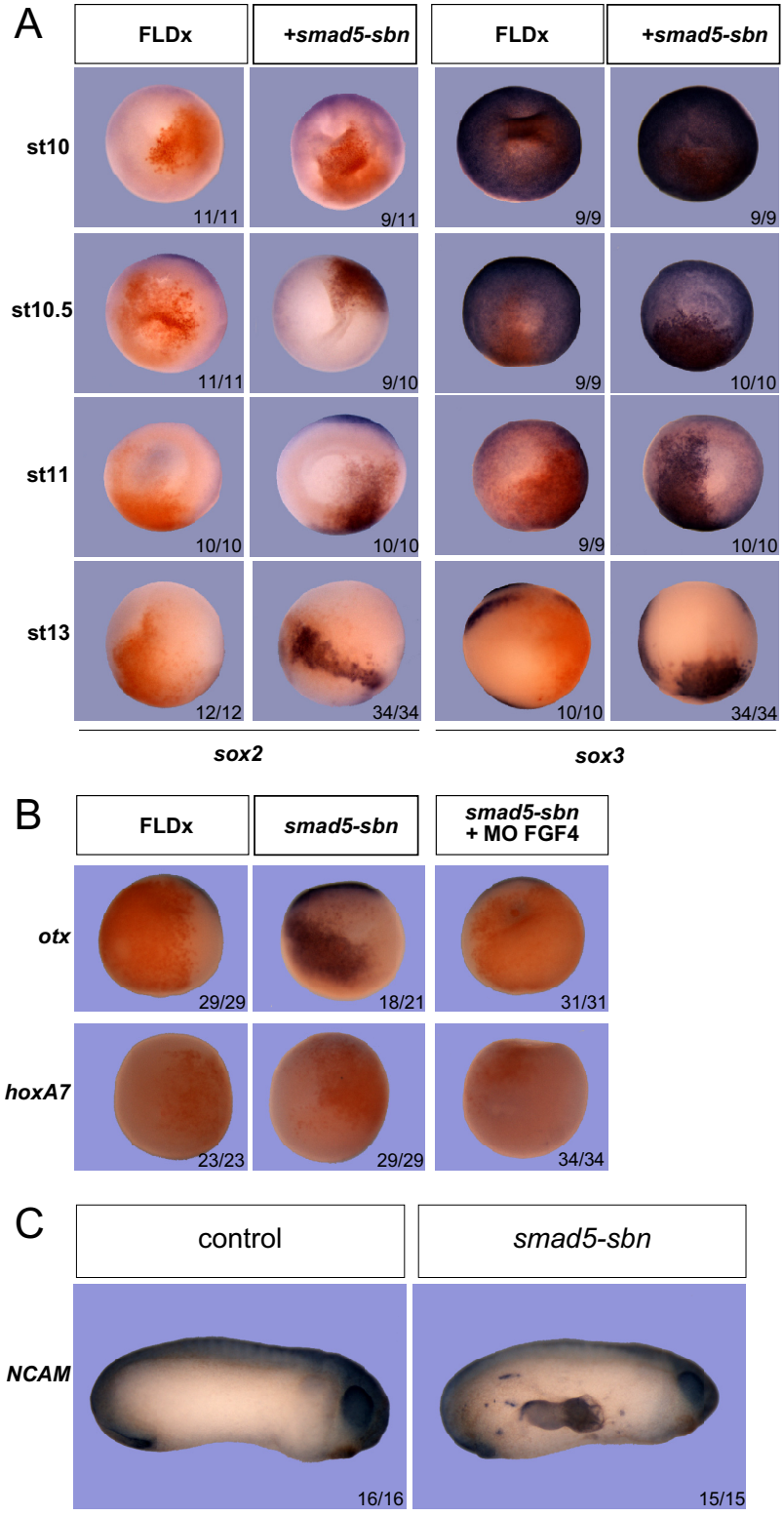

Fig. S1. In vivo induction of anterior neural tissue by Smad5-sbn. (*A*) Embryos were injected in one AB4 blastomere at 16-cell stage with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA as indicated. Both *sox2* and *sox3* activation is detectable at stage 10.5. Note that the maternal message for *sox3* is uniformly distributed in the animal region and is cleared by stage 11. All embryos are viewed from the animal pole, dorsal to the top. (*B*) Ventral views of stage 13 embryos injected in AB4 at 16-cell stage with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA, and 23 ng FGF4 MO, as indicated. *Otx2* but not *hoxA7* is activated by Smad5-sbn, in an FGF4-dependent manner. (*C*) Lateral views of tailbud stage embryos injected in one AB4 blastomere at 16-cell stage with 3 ng *smad5-sbn* mRNA. The control embryo was uninjected. The neural tissue induced by Smad5-sbn expressed the late neural marker NCAM.

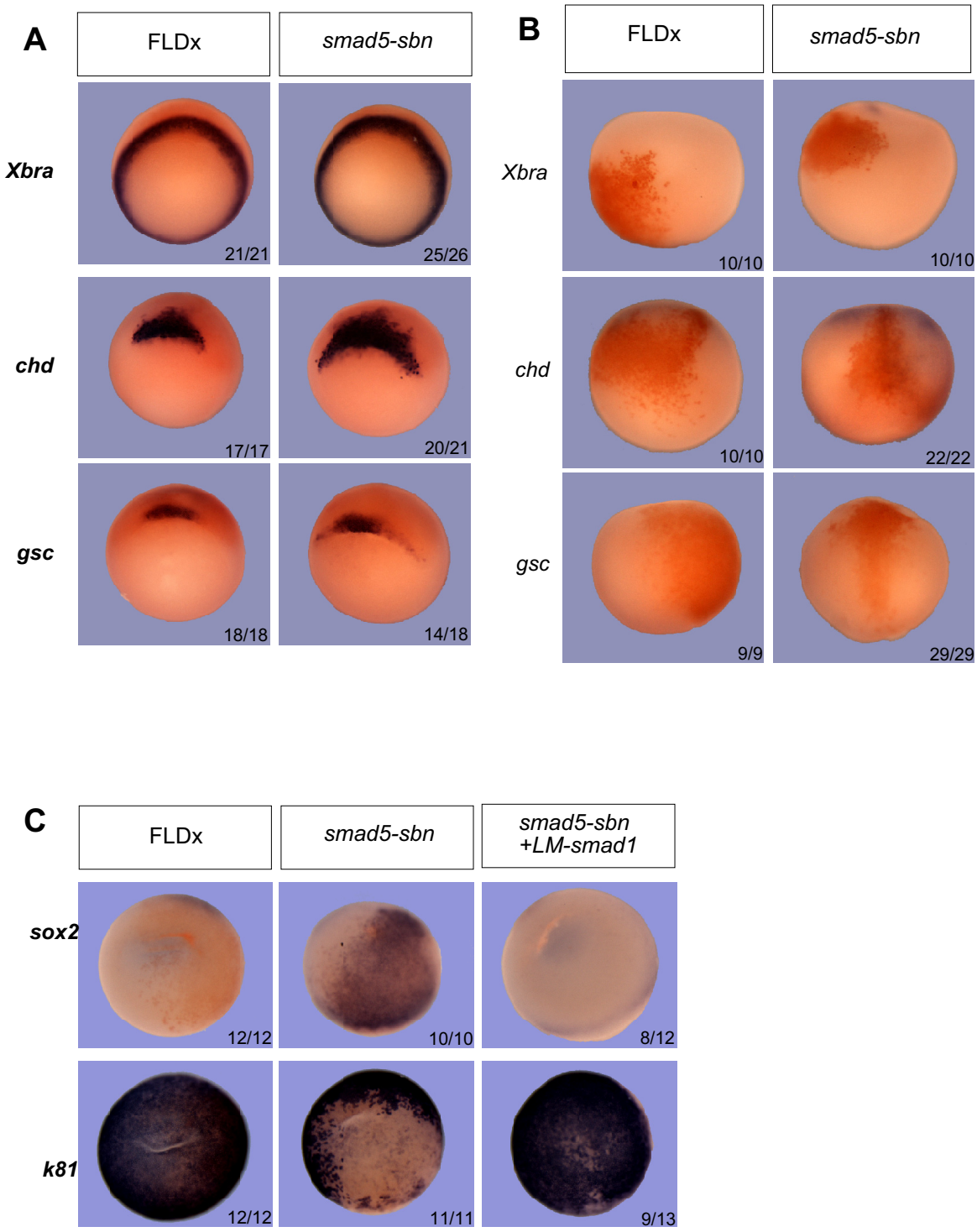

Fig. S2. Smad5-sbn inhibits BMP but not Activin/Nodal, or Wnt signaling. (*A*) Embryos were injected at the 4-cell stage in the 2 dorsal blastomeres with 2.5 ng/blastomere FLDx alone, or with 3 ng/blastomere *smad5-sbn* mRNA as indicated. The Activin/Nodal targets *Xbra*, *chd*, and *gsc* were not suppressed, ruling out an effect of Smad5-sbn on this pathway. The canonical Wnt targets *chd* and *gsc* were not suppressed, ruling out an effect of Smad5-sbn on this pathway. The organizer markers *chd* and *gsc* were expanded laterally, as expected from the reduction of BMP signaling. All embryos are viewed from the vegetal pole, dorsal to the top. (*B*) Sixteen-cell embryos were injected in one AB4 blastomere with 2.5 ng FLDx alone or with 3 ng *smad5-sbn* mRNA as indicated. None of the dorsal mesoderm markers were activated, ruling out indirect neural induction in response to Smad5-sbn. Embryos are viewed ventrally, anterior to the top. (*C*) 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*, and 250 pg *LM-Smad1* mRNAs, as indicated. LM-Smad1 reverted the effect of Smad5-sbn, indicating that the latter repressed BMP/Smad1 activity.

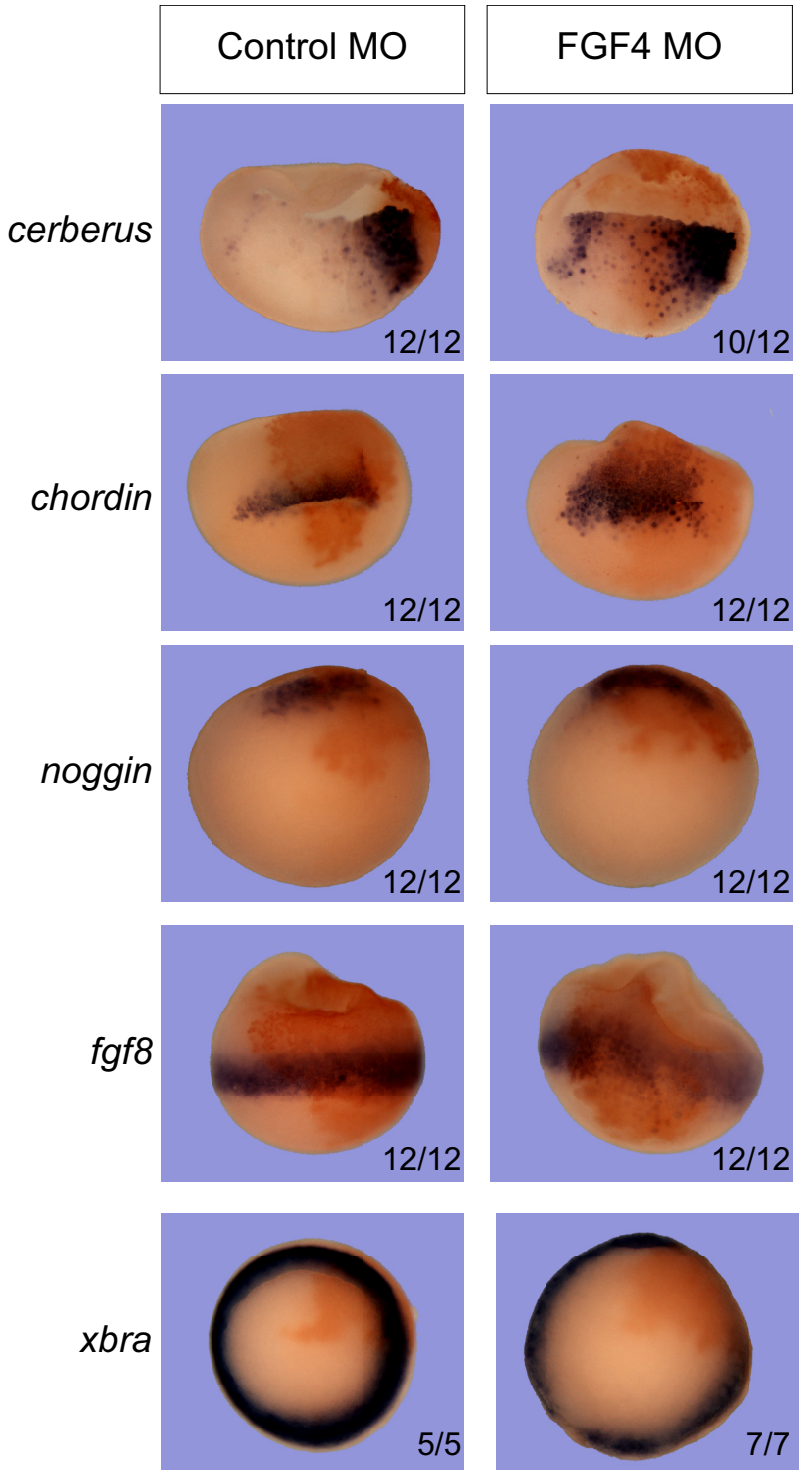

Fig. S3. FGF4 knockdown does not suppress expression of organizer-specific BMP antagonists. Four-cell embryos were injected marginally in one of the 2 dorsal cells with 2.5 ng FLDx and 23 ng control MO or 23 ng FGF4 MO as indicated. WISH analysis was performed at early gastrula stage 10. For *cerberus*, embryos were bisected before WISH. They are viewed dorsal to the right and animal to the top. For *chordin* and *FGF8*, embryos are viewed dorsally, animal to the top. For *noggin* and *Xbra*, embryos are viewed vegetally, dorsal to the top.

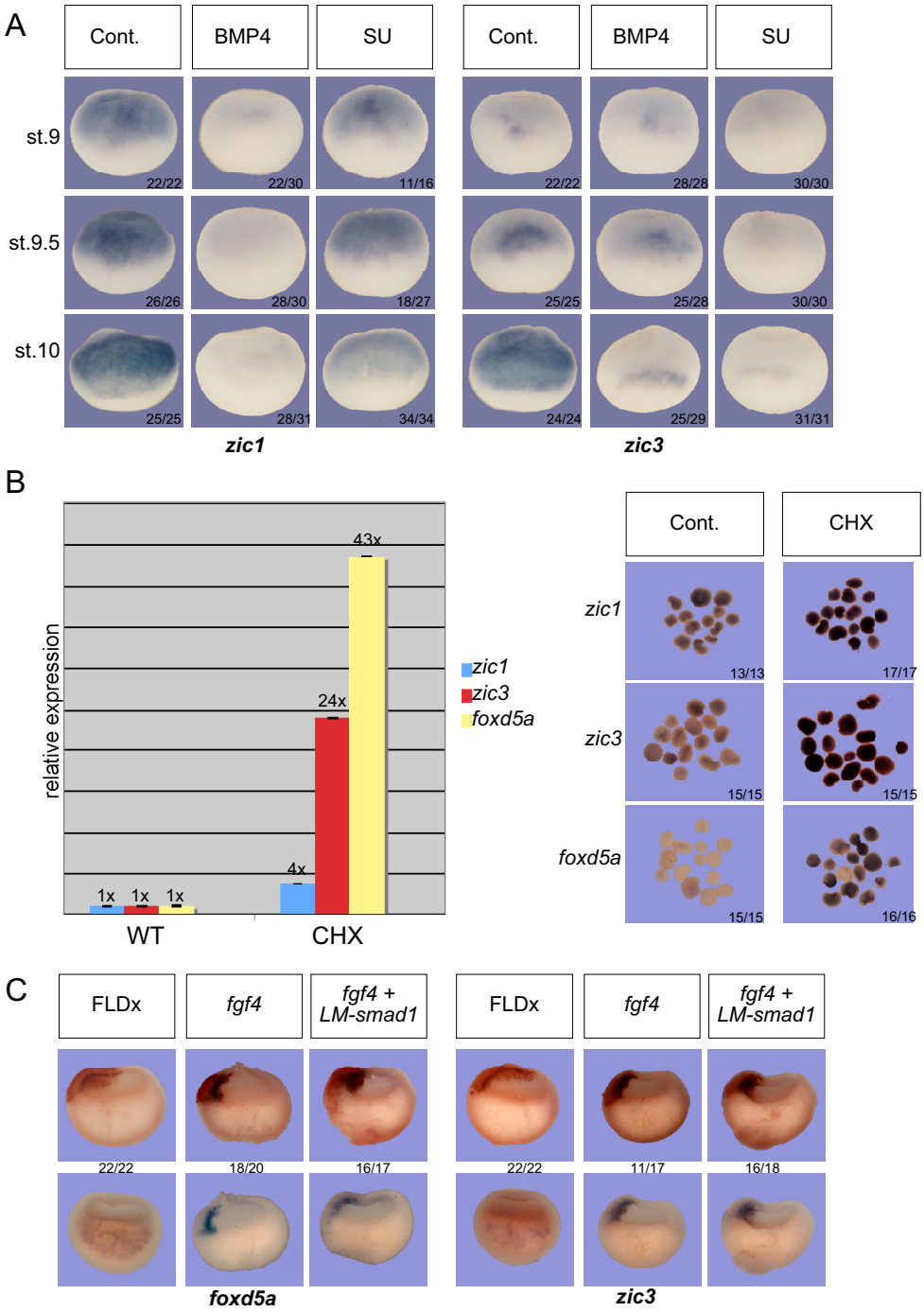

Fig. S4. Initiation of *zic1*, *zic3*, and *foxD5a* expression by BMP inhibition and FGF signaling. (*A*) Embryos were injected with 40 ng BSA at blastula stage 8, alone or in combination with 2 ng recombinant BMP4 protein. SU5402 treatment (180 μM) was initiated at the 4-cell stage. Embryos were harvested at the indicated stages and analyzed by WISH. The initiation of *zic1* depends on BMP inhibition, whereas the initiation of *zic3* depends on FGF signaling. Both genes are eventually down-regulated by either treatment at the onset of gastrulation (stage 10). All embryos are viewed dorsally, animal to the top. (*B*) Animal caps were prepared from stage 8 embryos and cultured in the presence or not of CHX (10 μg/mL) for a period of 2.5 h at 18 °C, when they were harvested. (*Left*) Quantitative RT-PCR analysis showing the normalized expression of *zic1*, *zic3*, and *foxD5a* in CHX caps (ODC was used as an internal control), relative to untreated control caps. (*Right*) WISH staining. Note that CHX activates all 3 genes. (*C*) Sixteen-cell embryos were injected in one AB4 blastomere with 2.5 ng FLDx alone or with 0.16 pg *FGF4*, and 250 pg *LM-Smad1* mRNAs, as indicated. WISH was performed on embryos fixed at stage 8.5. Embryos were hemisected following staining to improve imaging. (*Bottom*) Orange FLDx staining was cleared to better reveal the blue WISH staining. Both *foxD5a* and *zic3* were activated by FGF4, irrespective of the presence of LM-Smad1.

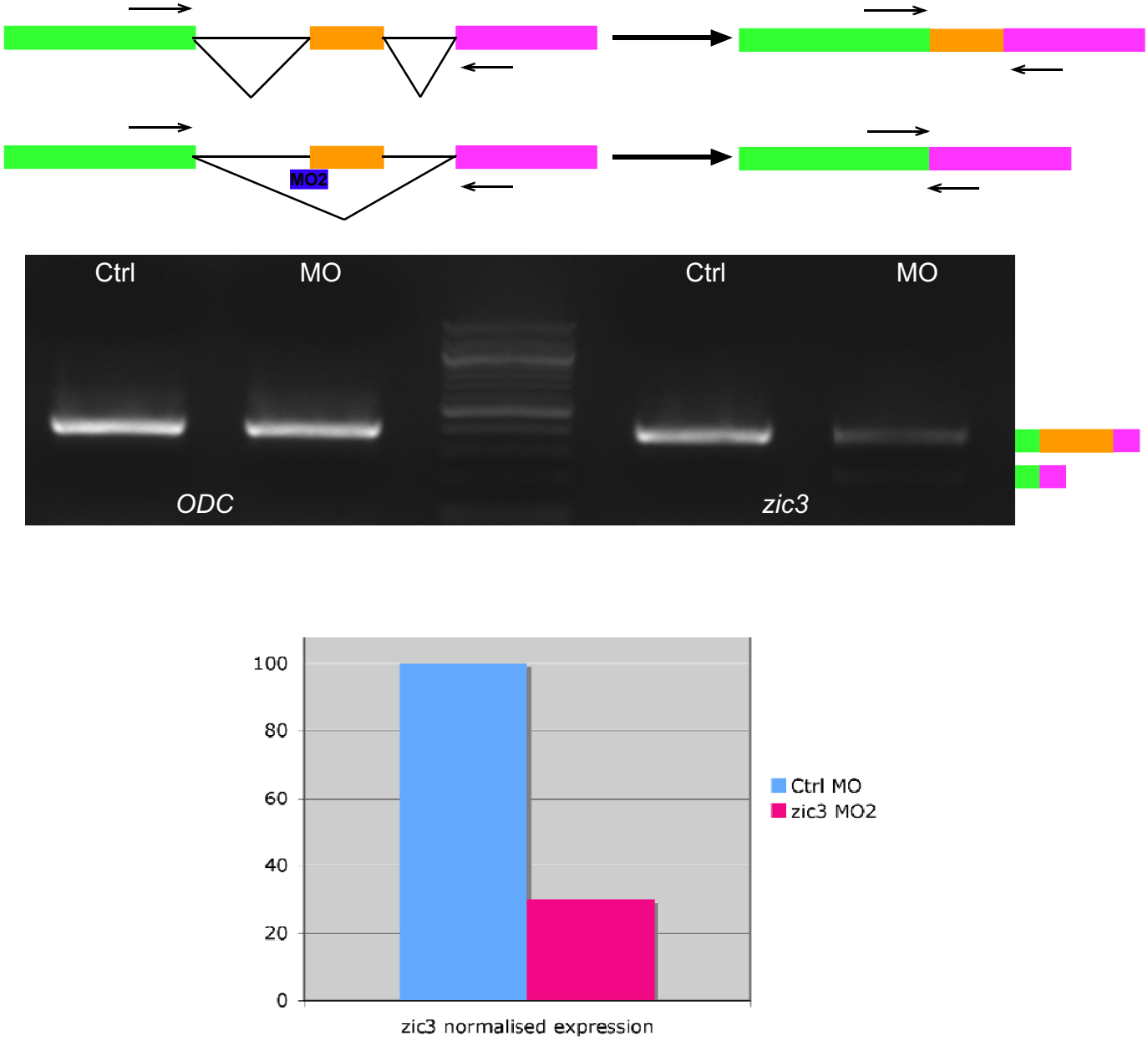

Fig. S5. Zic3 MO2 provokes exon 2 skipping. *Top*: Structure of the *zic3* pre mRNA (*Left*) and the mature mRNA following normal splicing (*Right*). The presence of Zic3 MO2 is predicted to provoke skipping of exon 2, yielding a shorter mRNA. The primers used to detect exon 2 are represented by arrows. (*Middle*) Ethidium bromide-stained gel of PCR products amplified from cDNAs prepared from total RNA extracted from stage 10 embryos injected in all cells at 4-cell stage with 23 ng/blastomere control MO or Zic3 MO2. ODC served as an internal control. (*Bottom*) Graph showing the amount of properly spliced *zic3* relative to the amount of ODC in control or Zic3 MO2 conditions. We used ImageJ software to quantify the intensity of the relevant bands on the gel shown above.

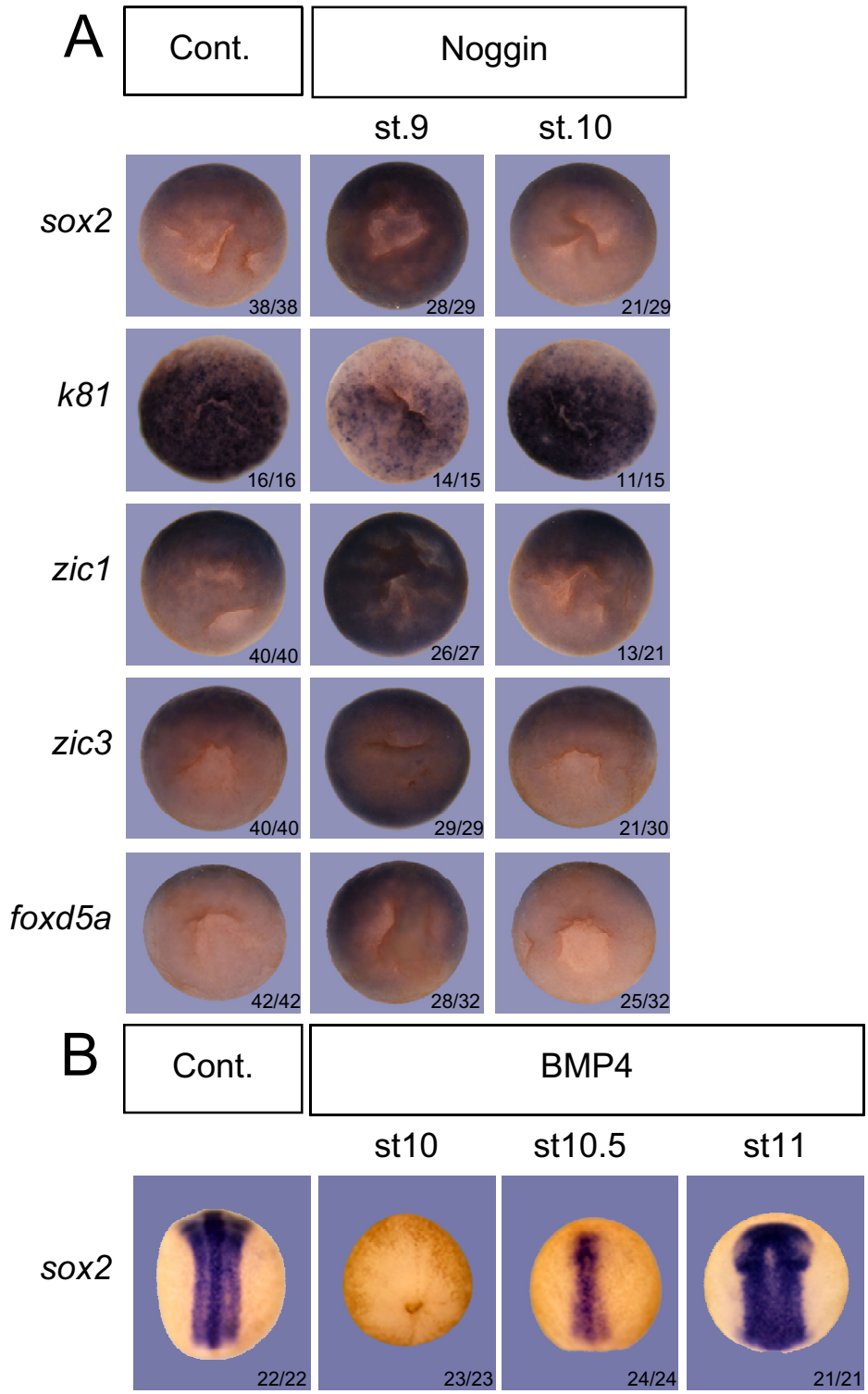

Fig. S6. A reversible neural state promoted by BMP inhibition before gastrulation. (*A*) Embryos were injected with 36 ng recombinant human Noggin protein at blastula stage 9 or at the onset of gastrulation (stage 10) as indicated, and analyzed at stage 10.5. Animal cells show reduced capacity to respond to Noggin after stage 9. All embryos are viewed animally, dorsal to the top. (*B*) Embryos received a blastocelic injection of 2 ng recombinant human BMP4 protein at the indicated developmental stages, and allowed to develop until early neurula stage. *sox2* expression is repressed by BMP4 exposure at the early gastrula stages 10 and 10.5. This effect is over by mid-gastrula stage 11, suggesting that neural cells are irreversibly engaged. In all panels, the number of embryos with normal *sox2* expression is indicated. All embryos are viewed dorsally, anterior to the top.

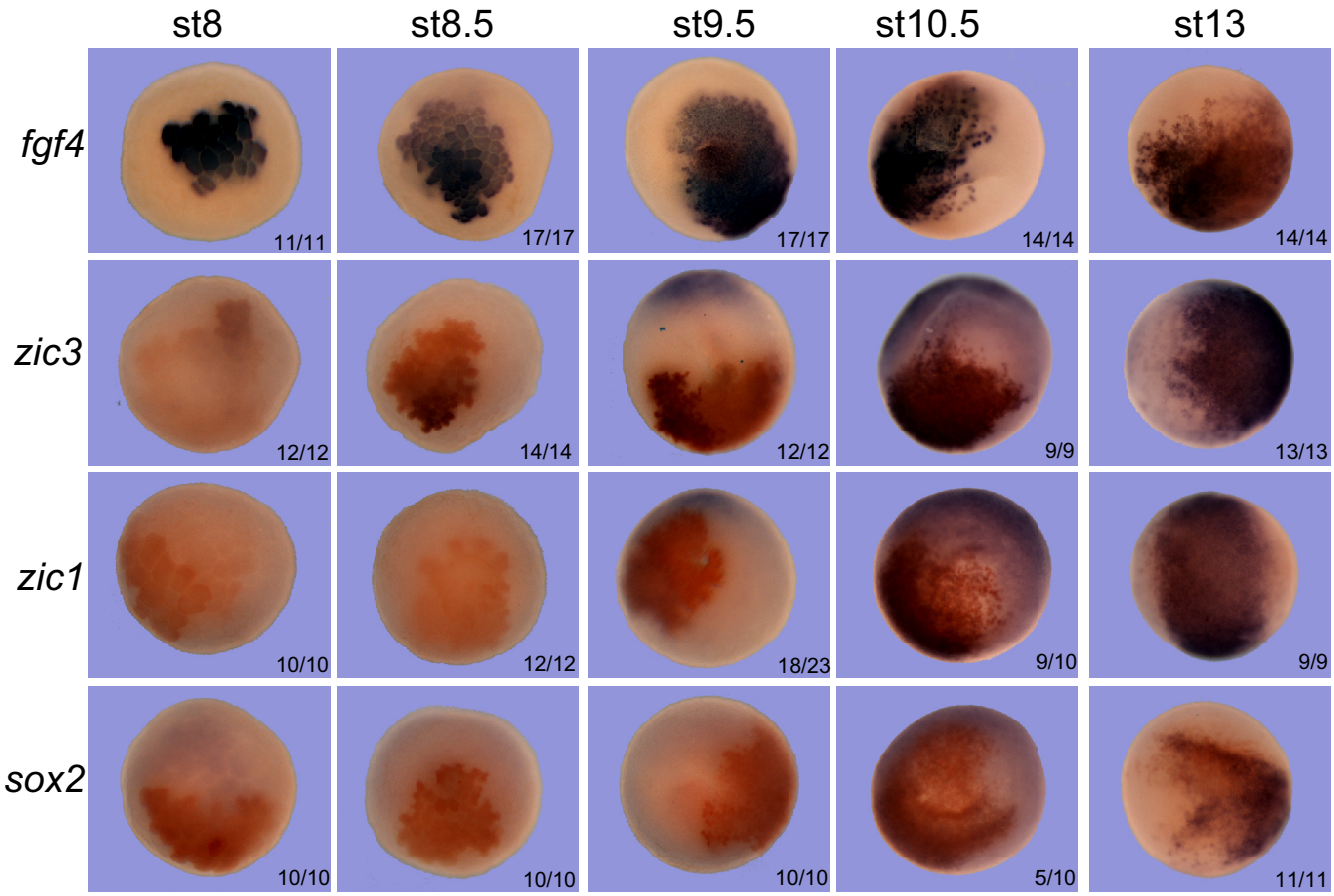

Fig. S7. Sequential activation of *FGF4*, *zic3*, *zic1*, and *sox2* in response to Smad5-sbn. Sixteen-cell embryos were injected in one AB4 blastomere with 2.5 ng FLDx and 3 ng *smad5-sbn* mRNA and fixed at various stages as indicated. All views are animal, except at stage 13, at which embryos are viewed ventrally. *FGF4* is the first gene activated by Smad5-sbn, as early as stage 8 (i.e., MBT), followed by *zic3* and *zic1*, whereas *sox2* is activated last, after the onset of gastrulation.