Supporting Information

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SI Experimental Procedures

Morpholino (MO) Sequences. For Cerberus MO we used a mix of the following morpholinos: 5′- GAAATATTCTTACCTGAGTGAAGGG-3′ (35 ng) and 5′-ACTTGCTGTTCCTGCACTGTGC-3′ (15 ng as in ref. 1), for a total of 50 ng/embryo; Lefty MOs were a 1:1 mix of the following morpholinos (25 ng each): 5′-GTAGTGACACCCATTCTGATGTGAC-3′ and 5′-GTAGTGACACCCATTCTGATGTGAC-3′ (as in ref. 2); ADMP MO was 5′-GGTCCATCTCATCAAGCTGCAGCTC-3′ (as in ref. 3); BMP4 MO was 5′-CAGCATTCGGTTACCAGGAATCATG-3′; (as in ref. 4); BMP7 MO was 5′-TTACTGTCAAAGCATTCATTTTGTC-3′; (as in ref. 4); miR-15/16 MO were as previously reported (5); and control MO (nontargeting morpholino) was 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

Luciferase Assays. HepG2 were cultivated in MEM 10% FCS supplemented with NEA. HEK293T were cultivated in DMEM 10% FCS. Plasmids were transfected using Transit-LT1 (MirusBio) and, after 24 h, the medium was changed to 0.2% FCS. If using siRNAs or miRNAs, cells were transfected with lipofectamin RNAi-MAX (Invitrogen) the day before DNA transfection. Cells were harvested 48 h after DNA transfection. The luciferase reporter *ID1*-BRE-lux (6) was cotransfected with *CMV–β-gal* to normalize for transfection efficiency by chlorophenol red-β-D-galactopyranoside (CPRG) (Roche) colorimetic assay. DNA content in all samples was kept uniform by adding *pCS2* plasmid. Each sample was transfected in duplicate. Each experiment was repeated at least twice. Mature miR-15 sequences were

wt sense 5′-UAGCAGCACAUAAUGGUUUGUGUU-3′;
wt antisense 5′-CACAAACCAuuAuGuGCuGGAuuu-3′.

ACVR2a *Silencer validated siRNA* was purchased from Ambion: sense 5′-GGACUGAUUGUGUAGAAAAtt-3′ as in ref. 5. Control scramble siRNA was as in ref. 6.

For experiments in *Xenopus* embryos, embryos were injected at the two- to four-cell stage (otherwise differently indicated) with 80 pg/embryo of luciferase reporter DNA together with 100 pg/embryo *lacZ* mRNA to normalize for injection volumes between different samples. Each sample was injected in at least 30 embryos, and the luciferase and β-galactosidase activities quantitated in groups of five gastrula-stage embryos to determine the experimental variability. Each experiment was repeated in at least two independent batches of embryos.

qRT-PCR. Poly(A)$^+$-RNA was retrotranscribed with M-MLV reverse transcriptase (Invitrogen) and oligo-d(T) primers following total RNA purification with TRIzol (Invitrogen). Real-time PCR messengerRNAs were performed on a RotorGene 3000 (Corbett) using the FastStart SYBR Green Master Mix (Roche). Sequences of primers were as follows:

EF1α fwd: 5′-CCTGAACCACCCAGGCCAGATTGGTG-3′
EF1α rev: 5′-GAGGGTAGTCAGAGAAGCTCTCCACG-3′
xDkk-1 fwd: 5′-ACCAAGCACAGGAGGAAAGG-3′
xDkk-1 rev: 5′-GGTTCAGGGAAGACCAGAGC-3′
Cer fwd: 5′-TGGCAGTAAAGCACAGGAAA-3′
Cer rev: 5′-GCAAGCAATGGGAACAAGTA-3′.

1. Kuroda H, Wessely O, De Robertis EM (2004) Neural induction in Xenopus: Requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. *PLoS Biol* 2:E92.
2. Cha YR, Takahashi S, Wright CV (2006) Cooperative non-cell and cell autonomous regulation of Nodal gene expression and signaling by Lefty/Antivin and Brachyury in Xenopus. *Dev Biol* 290:246–264.
3. Reversade B, De Robertis EM (2005) Regulation of ADMP and BMP2/4/7 at opposite embryonic poles generates a self-regulating morphogenetic field. *Cell* 123:1147–1160.
4. Reversade B, Kuroda H, Lee H, Mays A, De Robertis EM (2005) Depletion of Bmp2, Bmp4, Bmp7 and Spemann organizer signals induces massive brain formation in Xenopus embryos. *Development* 132:3381–3392.
5. Martello G, et al. (2007) MicroRNA control of Nodal signalling. *Nature* 449:183–188.
6. Dupont S, et al. (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. *Cell* 136:123–135.

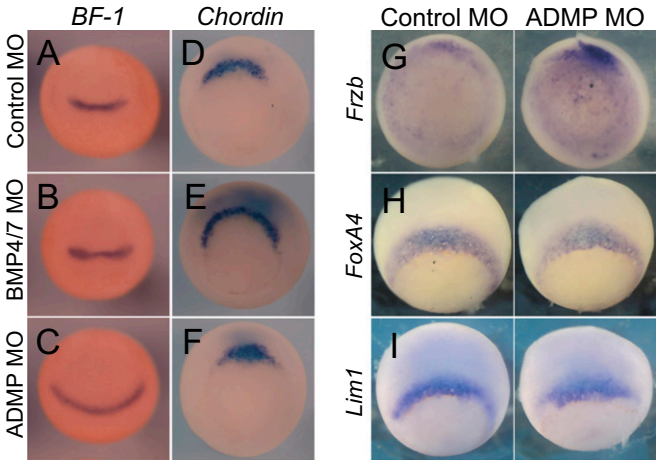

Fig. S1. (*A–F*) Expression pattern of *BF-1* (*A–C*) and *Chordin* (*D–F*) of embryos injected as Fig. 1 *A–I*. (*G–I*) Expression pattern of the indicated markers of embryos injected as in Fig. 1 *Q–T*. Pictures are the representatives of >20 embryos from two independent experiments. Frequency of the shown phenotype was >80%.

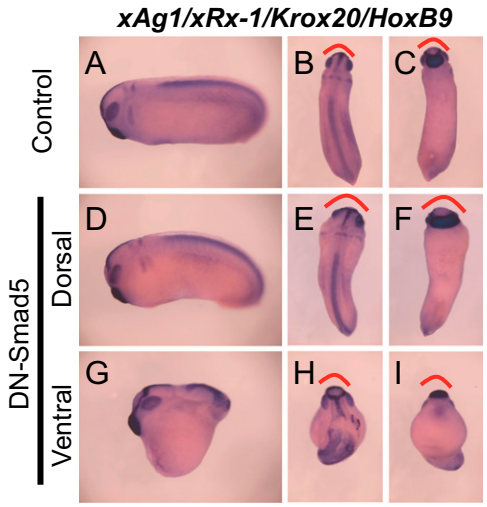

Fig. S2. (*A–I*) Embryos were injected with *dominant-negative Smad5* mRNA (DN-Smad5; 100 pg/embryo) into the marginal zone of dorsal (*D–F*) or ventral (*G–I*) blastomeres at the four-cell stage and expression pattern of neural genes (x*Ag1*, *xRx-1*, *Krox20*, and *HoxB9*) were visualized by in situ hybridization at tail bud stage. Note that the anterior structure is expanded in dorsally injected embryos (red brackets). (*A*, *D*, and *G*) Lateral view. (*B*, *E*, and *H*) Dorsal view. (*C*, *F*, and *I*) Ventral view. Pictures are the representatives of >20 embryos from two independent experiments. Frequency of the shown phenotype was >85%.

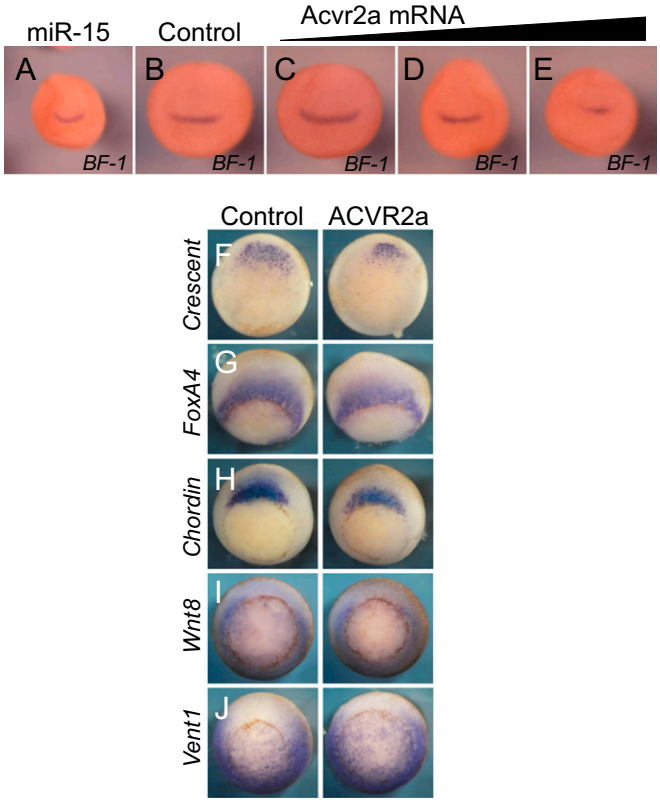

Fig. S3. (*A–E*) *BF-1* expression of the embryos injected as Fig. 3 *B–F* and harvested at early neurula stage. Numbers of embryo $n > 18$, frequency of the shown phenotype >70%. (*F–J*) Expression at gastrula stage of the indicated markers of embryos radially injected with *ACVR2a* (100 pg) mRNA.

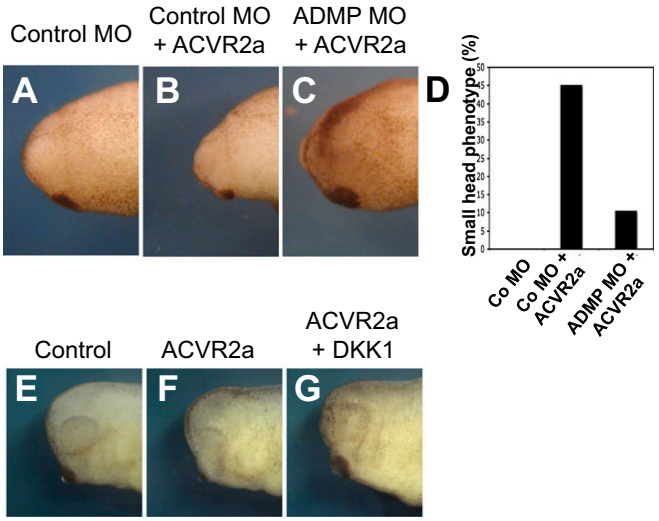

Fig. S4. Embryos were radially injected with indicated MOs and mRNA and head/anterior structure was examined at late neurula stage. (*A*–*C*) Close-up picture of head/anterior phenotype. (*D*) Quantification of frequency of small head phenotype. Number of embryos examined are 26 for control morpholino (CoMO), 32 for CoMO + ACVR2a, 31 for ADMP MO + ACVR2a. (*E*–*G*) Close-up pictures of head/anterior phenotypes at late neurula stage of embryos injected with the indicated mRNAs. Number of embryos (*n*) was ≥25, derived from two independent experiments; representative pictures are shown. *ACVR2a* (100 pg) mRNA; *hDKK1* (10 pg) mRNA.

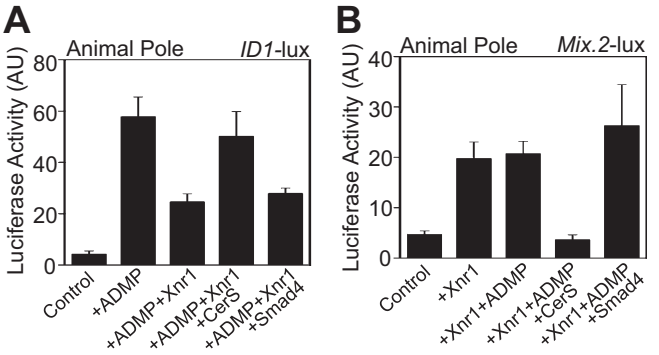

Fig. S5. (*A* and *B*) Embryos were injected in the animal pole with either *ID1*-luciferase reporter or Mix.2-luciferase reporter in combinations with *ADMP* (100 pg/embryo), *Xnr1* (100 pg/embryo), *CerS* (100 pg/embryo), and *Smad4* (100 pg/embryo) mRNAs. Data are given as mean and SD.

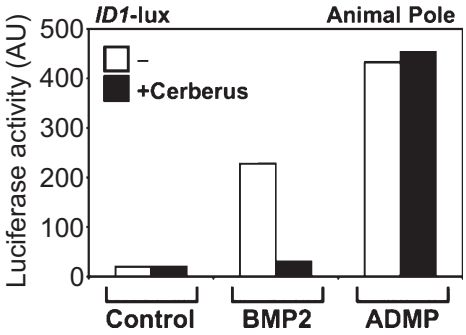

Fig. S6. Luciferase assay in *Xenopus* embryos as in Fig. 2*D*. mRNA doses were: *ADMP* and *BMP2* (200 pg/embryo); *Cerberus* (100 pg/embryo).

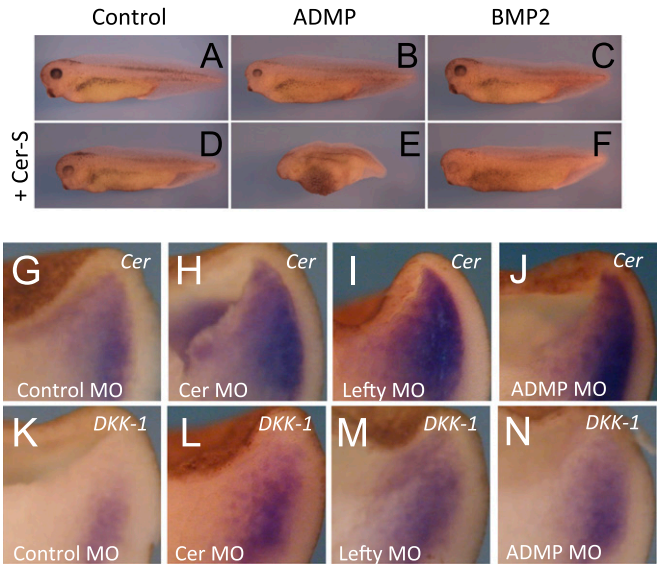

Fig. S7. (*A–F*) Embryos injected as Fig. 4 *A–F*, but in suboptimal doses (*ADMP* mRNA 5 pg/embryo, *BMP2* mRNA 5 pg/embryo, *CerS* 30 pg/embryo) were grown to tadpole stage (higher doses of *Cer-S* mRNA induce cyclopia) (1). Frequency of embryos lacking eyes were (*J*) 0% $n = 20$, (*K*) 0% $n = 22$, (*L*) 0% $n = 22$, (*M*) 0% $n = 21$, (*N*) 60% $n = 20$, (*O*) 0% $n = 21$. (*G–N*) Expression of head-organizer genes *Cerberus* (*Cer*) and *DKK-1* in hemisectioned embryos injected with the indicated MOs (50 ng/embryo). Note that upon depletion of Nodal antagonists the anterior border of the head organizer expands toward the anteriormost endoderm, phenocopying loss of ADMP.

1. Kuroda H, Wessely O, De Robertis EM (2004) Neural induction in Xenopus: Requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. *PLoS Biol* 2:E92.

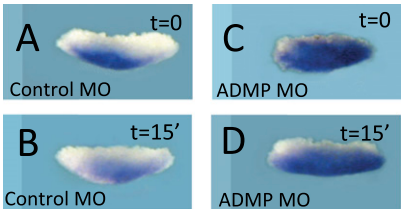

Fig. S8. (*A–D*) In situ hybridization for *Cerberus* on anterior endoderm explants (the explants were cut from the Brachet cleft to the bottom of the embryo) removed from embryos injected with the indicated morpholinos. Explants were fixed after dissection ($t = 0$) or cultured for 15 min before harvesting ($t = 15$ min).

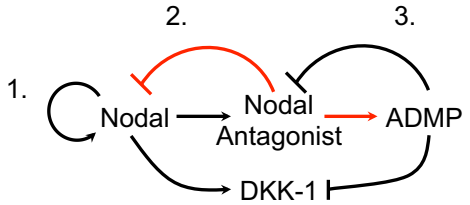

Fig. S9. Scheme illustrating the circuits from which the head-inducing network is assembled. (*1*) Positive feedback in Nodal expression. (*2*) Negative feedback between Nodal and Nodal antagonists (1). (*3*) As described in this paper, Nodal antagonists facilitate ADMP signaling that, in turn, negative feedbacks on their expression, all together establishing a self-regulating loop. Red lines indicate the extracellular regulations, whereas black lines stand for transcriptional regulations.

1. Piccolo S, et al. (1999) The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397:707–710.