Supporting Information

Goto et al. 10.1073/pnas.1008954107

SI Materials and Methods

Cloning of *Xenopus furry* and Construction. The following primers were used in this study:

5′-CCGCCAAGGCATGCTTGATATCTTTGTCCT-3′ and 5′-ACTTCTAAGAAGTAATGGGCGCATTCCTGC-3′ for 5′-RACE;

Forward 5′-TATACAAGACATGGCCAGCC-3′ and reverse 5′-TCTGCGTAGCAGTATTGCTT-3′ to obtain the fragment 197–1488;

Forward 5′-GTTGCACTGGAATCCTTGTA-3′ and reverse 5′-AAGCTTTCAAGTATGGAGGGACTAAGCTGATCC-AAAACATCAACCATAGG-3′ to obtain the fragment 1410–2599;

Forward 5′-ATTCTCAAAGAAGTACGGGC-3′ and reverse 5′-CTTCCAATGTCAAGAGCACT-3′ to obtain the fragment 2490–5949;

Forward 5′-CTTAGGGCCCTTAAGCAGCCTCTGTCTG-CCCACGCCTTATCTGACCTTCTCTCCAGATTGGT-GGAGGTTATAGG-3′ and reverse 5′-CCTGAACTG-CTGCTCGCTGTTTGTGT-3′ to obtain the fragment 5820–7568;

Forward 5′-GGCTCAGCAAAAACCCTTCT-3′ and reverse 5′-TCAGAGATTAGTGCCGGAAACGCT-3′ to obtain the fragment 7426–9239.

Each partial cDNA fragment was joined using PCR to obtain the full-length *Xenopus furry* (*Xfurry*).

Each construct was made by PCR using the following primers (restriction enzyme sites are underlined):

5′-GGGAATCGATTATACAAGACATGGCCAGCC-3′ for the forward primer of N-terminal (NT) constructs;

5′-CCCGAATTCTCATTCCTCCATTGTCTGGATGG-3′ for the reverse primer of NT1;

5′-CCCGAATTCTCATTCCTCCATTGTCTGGATGG-3′ for the reverse primer of NT2;

5′-GGGGGGAATTCCTATATGACTATTTTCCATTGGG-3′ for the reverse primer of NT3;

5′-CCCCGTAATTCTCACGTAATCATGTCTTCAGGCT-3′ for the reverse primer of NT4;

5′-AGGGATCGATCATCCAGACAATGGAGGAAC-3′ for the forward primer of C-terminal 1 (CT1);

5′-GGGAATCGATCAACCAGCTCATGTCTGACT-3′ for the forward primer of CT2;

5′-GGGGATCGATATGGGAGACTCGGAAGAAAAAC-3′ for the forward primer of CT3;

5′-CCCCACTAGTTCAGAGATTAGTGCCGGAAA-3′ for the reverse primer of CT1 and CT2;

5′-GGGGGAATTCTCAGAGATTAGTGCCGGAAA-3′ for the reverse primer of CT3, the C terminus of engrailed repressor (enR) + CT3 and *herpes simplex virus protein* (VP16) +CT3;

5′-AGTTCCAGTTGTTTTTCTTCCAGAAGCGTCTGGT-GCGTAT-3′ for the reverse primer of the N terminus of NT3+CT3;

5′-ATACGCACCAGACGCTTCTGGAAGAAAAACAACT-GGAACT-3′ for the forward primer of the C terminus of NT3+CT3;

5′-TCGAGTTACTTGCTGATGCCatgagtaaaggagaagaact-3′ for the reverse primer of the N terminus of NT3 +GFP;

5′-agttcttctcctttactcatGGCATCAGCAAGTAACTCGA-3′ for the forward primer of the C terminus of NT3+GFP;

5′-AGTTCTTCTCCTTTACTCATGAGATTAGTGCCGG-AAACGC -3′ for the reverse primer of the N terminus of CT3+GFP;

5′-GCGTTTCCGGCACTAATCTCATGAGTAAAGGAGA-AGAACT-3′ for the forward primer of the C terminus of Xfurry+GFP and CT3+GFP;

5′-GGGGGAATTCTCATTTGTATAGTTCATCCA-3′ for the reverse primer of GFP constructs;

5′-ATGGTTCTCTTCCTAGATCTTATGACTATTTTCCA-TTGGG-3′ for the reverse primer of the N terminus of NT3+Siamois homeodomain (SiaHD);

5′-CCCAATGGAAAATAGTCATAAGATCTAGGAAGA-GAACCAT-3′ for the forward primer of the C terminus of NT3+SiaHD;

5′-GGGGGAATTCTCATCTAGGTCCTTCTGAAGTTGG-GGATTT-3′ for the reverse primer of the C terminus of NT3+SiaHD;

5′-TATAATCGATACCGCCACCATGGCCCTGGAGGAT-CGCTG-3′ for the forward primer of the N terminus of enR+CT3;

5′-AGTTCCAGTTGTTTTTCTTCGGATCCCAGAGCAG-ATTTCT-3′ for the reverse primer of the N terminus of enR+CT3;

5′-TATAATCGATACCGCCACCATGGCCCCCCCGAC-CGATGT-3′ for the forward primer of the N terminus of VP16+CT3;

5′-AGTTCCAGTTGTTTTTCTTCCCCACCGTACTCGTC-AAT-3′ for the reverse primer of the N terminus of VP16+ CT3;

5′-GAAGAAAAACAACTGGAACT-3′ for the forward primer of the C terminus of enR+CT3 and VP16+CT3.

To confirm the specificity of *Xfurry* morpholino oligonucleotide (*Xfurry*-MO), we made *Xfurry-GFP* constructs containing 5′ sequences that could be targeted by *Xfurry*-MO. The sequence of the 5′-region of the *Xfurry-GFP* cDNA was 5′-agtgtgtgagcctcttacctg-cccgtctctacctccccccccgcctctatacaagacATGagtaaaggagaa-3′ (the targeted sequence is underlined; ATG is the first methionine). The sequence of the 5′-region of the *5-mis-Xfurry-GFP* cDNA was 5′-agtCtgtCagGctGttacctgcGcgtctctacctccccccccgcctctatacaagac-ATGagtaaaggagaa-3′.

RT-PCR. For the RT-PCR assay, the following primers were used:

Forward 5′-CATTGGCAGACGGTTTGATG-3′ and reverse 5′-CTCCCCAGTTGAAATTGTCC-3′ for *Xfurry*;

Forward 5′-GCAGGCACCCAACAAGATGAT-3′ and reverse 5′-CCAGATTCGGGGTGCAGAGT-3′ for *pintallavis*;

Forward 5′-CTCCAGCCACCAGTACCAGATC-3′ and reverse 5′-GGGGAGAGTGGAAAGTGGTTG-3′ for *Siamois*;

Forward 5′- GCTGAACTATTTGATTCCACC-3′ and reverse 5′- ATGGCTTGTATTCTGTGGGGC-3′ for *cerberus*.

Other primers have been described previously in ref. 1 and in the *Xenopus* Molecular Marker Resource (http://www.xenbase.org/common/). RT-PCR experiments were performed more than three times for each experiment.

Embryos, Microinjection of RNAs and MOs, and Microsurgery. To observe phenotypes and to extract RNA, mRNAs encoding *Xfurry* (2 ng/blastomere), other constructs (100–1,000 pg/blastomere), small RNAs (200–400 pg/blastomere), and *Xfurry*-MO (5–7.5 ng/blastomere) were microinjected. Cycloheximide (Wako) (10 μg/mL) or anisomycin (Sigma) (25 μg/mL) was used for treatment of embryos and animal caps. The preincubation started at stage 7, except for

injection of small RNAs and microRNAs, and animal caps in the solution were dissected at stage 8 and cultured until stage 10.

For RT-PCR using animal caps without cycloheximide and anisomycin treatment, mRNAs as described above as well as *β-catenin* (500 pg/blastomere), *vegetal T-box transcription factor* (*VegT*) (500 pg/blastomere), and *Xenopus nordal related-5 gene* (*Xnr5*) (10 pg/blastomere) were microinjected into the animal poles of two-cell embryos, and animal caps were dissected at stage 8 and cultured until stage 10. For RT-PCR using animal caps with cycloheximide treatment, mRNAs [*engrailed receptor* (*enR*) + *leucine zipper* (*LZ*)] (400 pg/blastomere) and [*herpes simplex virus protein* (*VP16*) + *leucine zipper* (*LZ*)] (400 pg/blastomere) were microinjected into the animal poles of two-cell embryos, and the cycloheximide treatment was performed as described above.

For RT-PCR using animal caps with cycloheximide treatment, small RNAs (100–200 pg/blastomere), microRNA *mir-15* (1 ng/blastomere), and control mutant *mir-15* (1 ng/blastomere) with Alexa 488-dextran (Molecular Probes) (100 pg/blastomere) as a tracer were microinjected into the four animal pole cells of 32-cell embryos in the presence of cycloheximide. Animal caps dissected at stage 8 under a fluorescent microscope and were cultured until stage 10. To observe the nuclear localization of GFP-conjugated proteins, each construct was microinjected into the marginal zones of dorsal blastomeres of four-cell embryos. The mesodermal cells were dissected at stage 10 and stained with DAPI solution (2 μg/mL) for 20 min. The developmental stages were as defined by Nieuwkoop and Faber (2).

To confirm the specificity of *Xfurry*-MO, GFP constructs containing 5′ sequences of *Xfurry* and Alexa 594-dextran (Molecular Probes) (300 pg/blastomere) were injected as tracers into the animal poles of embryos at the four-cell stage, and fluorescence was observed at stage 10 (Fig. S3).

Western Blotting. Messenger RNA of *6Myc-Tag* (*6MYC*)-*GFP* (100 pg/blastomere) was coinjected into marginal zones of dorsal blastomeres of four-cell embryos with other *GFP* constructs. Total proteins were extracted from the dorsal sectors of the injected embryos at stage 10 and were fractionated into cytoplasm and nuclei as previously described, with modifications (3, 4). Extracted fractions were subjected to Western blotting using an anti-GFP antibody (mFX75; Wako). The signals of 6MYC-GFP were used for standardization.

1. Wylie C, et al. (1996) Maternal beta-catenin establishes a 'dorsal signal' in early *Xenopus* embryos. *Development* 122:2987–2996.
2. Nieuwkoop PD, Faber J (1967) *Normal Table of Xenopus laevis (Daudin)* (North Holland, Amsterdam).
3. Shimizu K, Gurdon JB (1999) A quantitative analysis of signal transduction from activin receptor to nucleus and its relevance to morphogen gradient interpretation. *Proc Natl Acad Sci USA* 96:6791–6796.
4. Shimizu K, Shirataki H, Honda T, Minami S, Takai Y (1998) Complex formation of SMAP/KAP3, a KIF3A/B ATPase motor-associated protein, with a human chromosome-associated polypeptide. *J Biol Chem* 273:6591–6594.

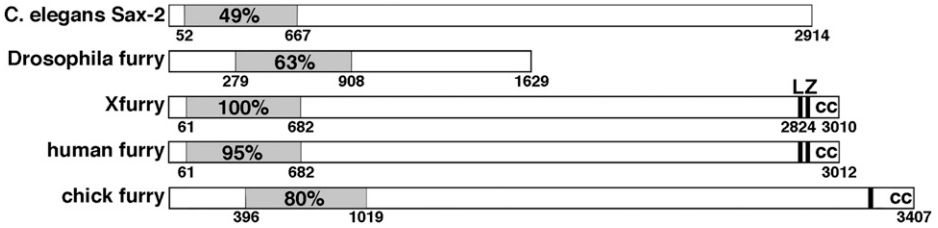

Fig. S1. Schematic of Xfurry and homologs. The percent amino acid identity of various furry homolog genes is indicated in the shaded box (FD). The number of amino acids in the domains, or length of the proteins, is indicated below each diagram. Black boxes indicate LZ motifs. CC, coiled-coil structure. Sax-2, sensory axon guidance gene.

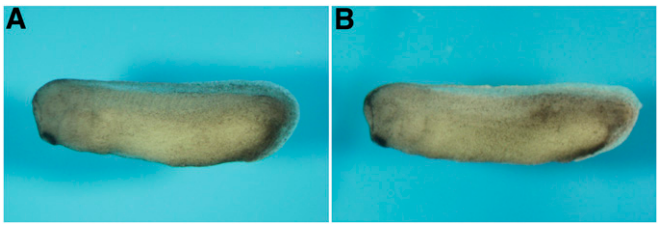

Fig. S2. Dorsal overexpression of *Xfurry*. (*A*) Control (stage 28). (*B*) Injection of *Xfurry* (2 ng/blastomere) into dorsal blastomeres of four-cell embryos had no effect on axis formation (n = 60).

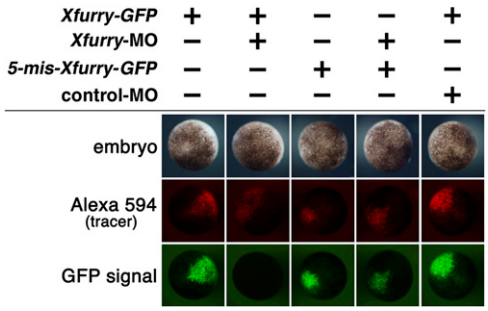

Fig. S3. Confirmation of the morpholino specificity. A tracer, Alexa 594-dextran (300 pg), GFP constructs, and morpholinos were injected in the animal pole of four-cell embryos and were observed at stage 10 to show the injected embryos (*Top Row*), the fluorescent Alexa 594-dextran tracer (*Middle Row*), and GFP fluorescence (*Bottom Row*). *Xfurry*-MO specifically reduced translation of sequences containing *Xfurry-GFP* targeted with *Xfurry*-MO at the 5′-region but did not reduce translation of *5-mis-Xfurry-GFP*, which has no targeted sequence.

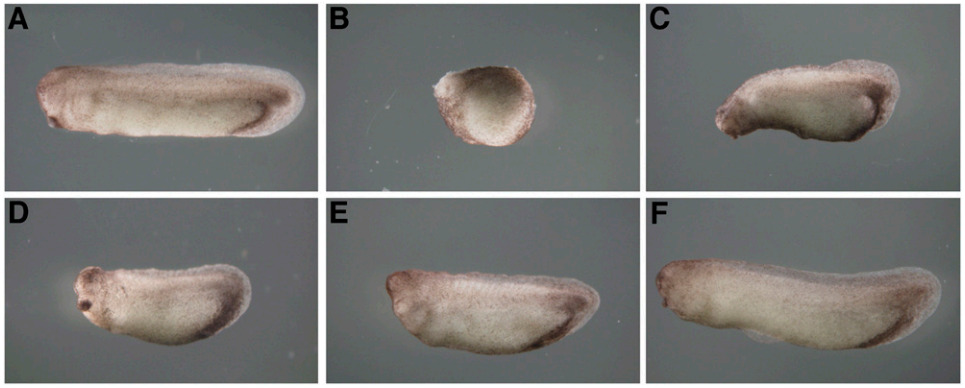

Fig. S4. Rescue experiments among *Xfurry*, *Xfurry*-MO, *enR+LZ*, and *VP16+LZ*. (*A*) Control (stage 33). (*B*) Injection of *Xfurry*-MO (5 ng/blastomere) into dorsal blastomeres of four-cell embryos interfered with head formation and shortened the axis of all embryos, as shown also in Fig. 1*K* ($n = 40$). (*C*) The cement gland was rescued, and the axis was slightly elongated by coinjection of *Xfurry* (2 ng/blastomere) and *Xfurry*-MO (5 ng/blastomere) into dorsal blastomeres of four-cell embryos (57.9%; $n = 57$). (*D*) Dorsal expression of *enR+LZ* (400 pg/blastomere) also rescued the phenotype of *Xfurry*-MO–injected embryos (78.6%; $n = 56$). (*E*) Dorsal injection of *VP16+LZ* (400 pg/blastomere) interfered with head formation and shortened the axis of all embryos, as in Fig. 2*G* ($n = 40$). (*F*) Dorsal expression of *Xfurry* (2 ng/blastomere) rescued the phenotype of *VP16+LZ*-injected embryos (52.5%, $n = 59$).

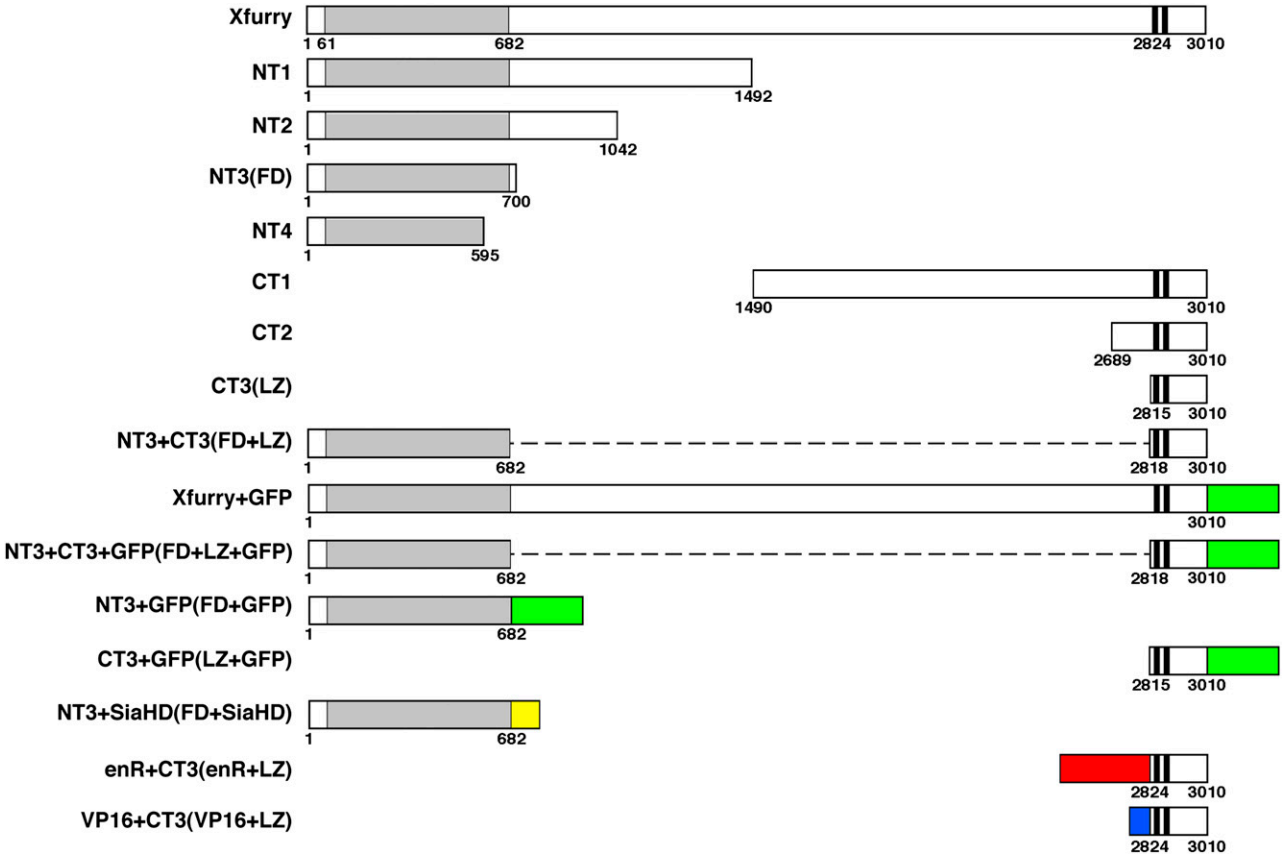

Fig. S5. Schematic of Xfurry and constructs. The furry domain is indicated in the shaded box. Black boxes indicate LZ motifs. The green boxes indicate GFP. The yellow box indicates the SiaHD. The red box indicates the enR domain. The blue box indicates the VP16 activator domain. FD, fuzzy domain.

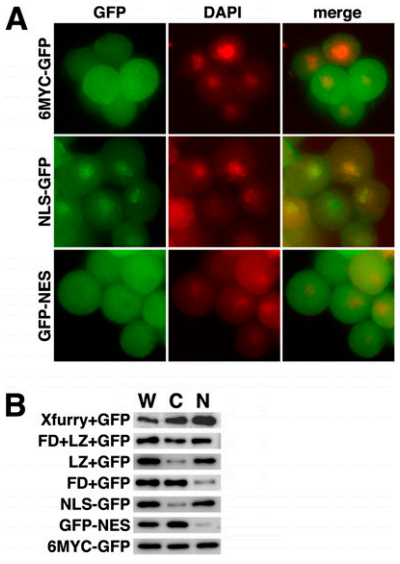

Fig. S6. Localization of Xfurry constructs with GFP tag. (*A*) Fluorescent signals of 6MYC-GFP, Nuclear-localized signal (NLS)-GFP, and GFP-nuclear export signal (NES) in mesodermal cells (stage 10). Red signals indicate cell nuclei stained with DAPI. Fluorescent signals of 6Myc-Tag (6MYC)-GFP were localized ubiquitously in both nuclei and cytoplasm. NLS-GFP and GFP-NES were localized in nuclei and cytoplasm, respectively. (*B*) Western blotting analysis of Xfurry constructs with GFP tag in mesodermal cells (stage 10). Messenger RNA of *6MYC-GFP* was coinjected with other *GFP* constructs. Each extracted fraction was subjected to Western blotting using an anti-GFP antibody and was standardized against 6MYC-GFP blotting. C, cytoplasm fraction; N, nuclear fraction; W, whole cells.

Fig. S7. Overexpression of nuclear DBF2-related 1 (NDR1) and NDR2. (*A*) Control (stage 33). (*B*) Overexpression of *NDR1* (1 ng/blastomere) into ventral blastomeres of four-cell embryos had no effect on axis formation (n = 60). (*C*) Ventral overexpression of *NDR2* (1 ng/blastomere) at the four-cell embryo stage also had no effect on axis formation (n = 60).

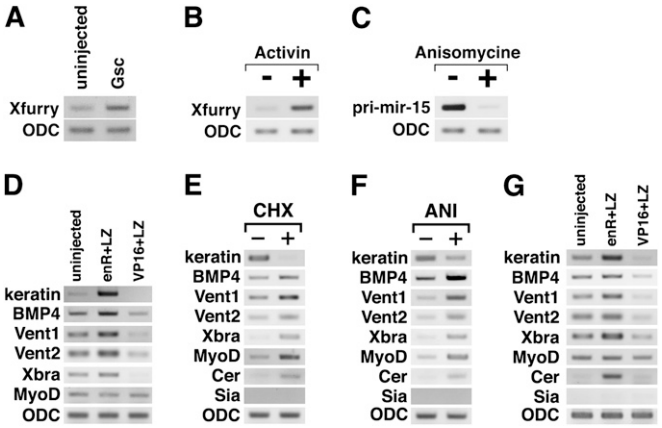

Fig. S8. RT-PCR analyses. (*A*) Overexpression of *goosecoid* (300 pg/blastomere) into all blastomeres of four-cell embryos increased *Xfurry* transcript levels (stage 10). Gsc, *goosecoid*; ODC, ornithine decarboxylase. (*B*) Animal caps dissected from stage-8 embryos were cultured in a solution including activin (10 ng/mL) until stage 10 and were assayed by RT-PCR. Expression of *Xfurry* was increased by the activin treatment. (*C*) The anisomycin treatment reduced expression of *primary miR-15* (*pri-miR-15*) (stage 10). (*D*) Expression of *enR+LZ* (400 pg/blastomere) induced epidermal, ventral, and mesodermal genes, and expression of *VP16+LZ* reduced those genes (stage 10). Expression of *myogenic differentiation* (*MyoD*) was not affected by *enR+LZ* or *VP16+LZ*. BMP4, bone morphogenetic protein 4; Vent1, VENT homeobox 1; Vent2, VENT homeobox 2; Xbra, Xenopus brachyury. (*E* and *F*) Superinduction in the animal caps treated with cycloheximide (CHX) (*E*) and anisomycin (ANI) (*F*) (stage 10). Expression of *keratin* was reduced by the treatments. Expression of *Siamois* (Sia) was not detected in the animal caps. Cer, *Cerberus*. (*G*) *enR+LZ* induced epidermal, ventral, mesodermal, and endodermal genes in the pre-midblastula transition cycloheximide-treated animal caps, and *VP16+LZ* reduced expression of those genes (stage 10). Expression of *MyoD* was not affected by *enR+LZ* and *VP16+LZ*.

Table S1. Induction of secondary axis by ventral injection of each construct

Construct	% embryos inducing secondary axis	No. of embryos
Xfurry, full-length	63.3	60
NT1	2.6	78
NT2	9.1	88
NT3 (FD)	28.1	64
NT4	0	58
CT1	0	60
CT2	0	59
CT3 (LZ)	0	60
NT3+CT3 (FD+LZ)	54.2	48