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Double assurance in the induction of axial development by egg dorsal determinants in *Xenopus* embryos

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Affiliations are included on p. 10.

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We recently reported that microinjection of *Xenopus nodal-related* (*xnr*) mRNAs into β-catenin-depleted *Xenopus* embryos rescued a complete dorsal axis. Xnrs mediate the signal of the Nieuwkoop center that induces the Spemann–Mangold organizer in the overlying mesoderm, a process inhibited by the Nodal antagonist Cerberus-short (CerS). However, β-catenin also induces a second signaling center in the dorsal prospective ectoderm, designated the Blastula Chordin and Noggin Expression (BCNE) center, in which the homeobox gene *siamois* (*sia*) plays a major role. In this study, we asked whether the Xnrs and Sia depend on each other or function on parallel pathways. Expression of both genes induced β-catenin-depleted embryos to form complete axes with heads and eyes via the activation of similar sets of downstream organizer-specific genes. Xnrs did not activate *siamois*, and, conversely, Sia did not activate *xnrs*, although both were induced by β-catenin stabilization. Depletion with morpholinos revealed a robust role for the downstream target Chordin. Remarkably, Chordin depletion prevented all ectopic effects resulting from microinjection of the mRNA encoding the maternal cytoplasmic determinant *Huluwa*, including the radial expansion of brain tissue and the ectopic expression of the ventral gene *sizzled*. The main conclusion was that the BCNE and Nieuwkoop centers provide a double assurance mechanism for axial formation by independently activating similar downstream transcriptional target gene repertoires. We suggest that Siamois likely evolved from an ancestral Mix-type homeodomain protein called Sebox as a *Xenopus*-specific adaptation for the rapid differentiation of the anterior neural plate in the ectoderm.

Huluwa | β-catenin | Chordin | Nodal | Siamois

Significance

Over a century ago, Hans Spemann described a process of double assurance during eye lens induction. We now reinvestigate this problem in *Xenopus* central nervous system (CNS) development. At blastula, the Nieuwkoop center in the endoderm induces the dorsal mesoderm, and the Blastula Chordin and Noggin Expression (BCNE) center in the dorsal ectoderm gives rise to the brain and floor plate. One is driven by *Xenopus Nodal-related* genes (*xnrs*) and the other by the homeobox gene *siamois*, yet both activate similar sets of downstream growth factor antagonists. We propose that the BCNE evolved in the plane of the ectoderm in *Xenopus* to facilitate the rapid induction of the neural plate by vertical signals emanating from the underlying endomesoderm at gastrula. CNS differentiation has belt and suspenders.

The amphibian embryo has provided important insights into the development of the vertebrate dorsal axis. The most famous experiment in embryology was performed by Hilde Mangold a century ago under the supervision of Hans Spemann (1). When the dorsal lip of the blastopore, the region where endomesodermal involution starts, was transplanted into the ventral side of the embryo, neighboring cells were induced to become the central nervous system (CNS) and dorsal mesoderm such as somites and kidney. This experiment defined embryonic cell–cell inductions as the central mechanism of vertebrate development (2). Once cloning became possible, studies showed that the gastrula organizer secretes a cocktail of BMP (Bone Morphogenetic Protein), Nodal, and Wnt antagonists (3, 4). Paradoxically, the organizer also secretes dorsal BMPs designated Anti-Dorsalizing Morphogenetic Protein (ADMP) and BMP2 (5–8). Biochemical studies of organizer proteins have revealed many novel molecular signaling mechanisms by secreted antagonists such as Noggin, Chordin (Chd), Dickkopf (Dkk1), Frzb, Cerberus, and Lefty/Antivin (9).

At the blastula stage, an earlier induction process occurs in which *Xenopus Nodal-related* genes (especially *xnr5* and *xnr6*) are transcribed very early in the dorsal (future back) and vegetal (future endoderm) regions of the embryo (10), activated by a combination of VegT, Vg1, and β-catenin signals (11). Pieter Nieuwkoop discovered that recombining explants of the vegetal cells with prospective ectodermal cells (animal cap) resulted in the induction of the mesoderm and that the dorsal endoderm induced the Spemann organizer mesoderm (12, 13). There are multiple *Nodal-related* genes in *Xenopus* which would be very difficult to inhibit individually, but fortunately, a fragment of the secreted Cerberus protein, called Cerberus-short (CerS), consisting of only the cystine knot, was found to be a specific inhibitor of Nodals, but not of other mesoderm-inducing TGF-β (Transforming Growth Factor β) family members (14). An exception among *xnrs* is *xnr3*, which is a primary response gene to β-catenin and encodes a mutated form that is unable to induce the mesoderm, and instead functions as a neural inducer by inhibiting BMP signals (15, 16). Using *cerS*-injected endodermal fragments, it was found that high levels of endogenous Nodals were required for dorsal organizer induction in recombinant explants while ventral mesoderm induction was

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mediated by low Nodal signals (17). In addition to *xnr5/6*, at the blastula stage, there is a second signaling center called the BCNE (Blastula Chordin and Noggin Expression) center in the dorsal-animal region of the embryo (18, 19). Expression of BCNE genes (*sia*, *chd*, *noggin*, and *xnr3*) is activated by β-catenin and overlaps in part with the Nieuwkoop center in the dorsal marginal zone (18, 20).

Formation of the organizer is a process that starts at the fertilized egg stage. An important discovery from zebrafish genetics was the *huluwa* (*hwa*) mutation that results in the complete loss of the dorsal organizer (21). Maternal mRNA for this gene is stored in the vegetal pole of the oocyte and translated in dorsal cells of the zebrafish blastoderm. Hwa encodes a transmembrane protein with a very short extracellular domain and a large cytoplasmic domain. Hwa protein elicits a powerful Wnt signal via the degradation of Axin1, an adaptor protein essential for the destruction of β-catenin (21). Stabilization of β-catenin on the dorsal side of the early blastula is the key element in dorsal development and requires a microtubule-mediated cortical rotation of egg cytoplasm in both fish and frog embryos (22, 23). Hwa activity also requires endolysosomal function in early development (24, 25). *Xenopus* embryos microinjected into the ventral side with components of the Wnt signaling pathway provide a simple assay system in which complete secondary axes with trunks and head structures containing the brain and two eyes develop. Many of the sequential steps in the canonical Wnt signal transduction pathway were identified by injecting mRNAs for *Wnt1*, Wnt receptors, the adaptor Dishevelled (Dvl), dominant-negative GSK3β, dominant-negative Axin1, and β-catenin in *Xenopus* embryos (26–28). Among these reagents, Hwa is unique in that it induces complete secondary axes with almost 100% penetrance (29).

We recently utilized *hwa*-injected embryos for an epistatic analysis of the self-organizing dorsal axis signaling pathway in *Xenopus* (29). We found that Hwa activity is inhibited by ventral BMP4 signals and that Wnt antagonists such as Dkk1 enhanced *hwa* dorsalization by inhibiting the late ventrolateral xWnt8 signal during gastrulation. In *Xenopus* embryology, an element that sometimes confounds nonspecialists is that at blastula, the early Hwa/β-catenin signal induces dorsal axis development (22) while at gastrula, the later xWnt8/β-catenin signal induces posteriorization: When the late xWnt8 signal is inhibited, for example, by injecting Dkk1 mRNA (27), enlarged head and brain structures are induced. Using β-catenin-depleted embryos, which provide a sensitized system, we showed that Nodals (Xnr1 or Xnr6) or their downstream target *chd* rescued complete axes including trunk-tail and large heads and eyes in a dose-dependent manner (29), even though in wild-type (WT) embryos, these mRNAs were only able to induce partial trunk-tail second axes. The question addressed here is whether the Nodal-driven Nieuwkoop center is the sole pathway for dorsal development.

In addition to Nodal signaling, early β-catenin signaling activates an immediate-early homeobox gene called *siamois* (*sia*)/*twin* (*sia1/2*) that when overexpressed induces complete secondary axis like *hwa* does (30–32). *sia* is expressed in the BCNE and Nieuwkoop centers starting at very early blastula stages (18, 33, 34). Sia is a transcriptional activator of the Paired homeobox family specific to amphibians. When an Engrailed repressor domain from *Drosophila* was fused to Sia, all organizer gene expression was eliminated including *goosecoid* (*gsc*), *noggin,* and *chd* (35, 36). We next asked what are the respective roles of Siamois and Xnrs in Spemann organizer formation? How are they regulated? Which target genes do they activate?

In the present study, we found that *siamois* or *xnr* mRNAs can independently induce complete dorsal axis development in embryos sensitized by β-catenin depletion. RNAseq analyses showed that both signals activate similar sets of target genes. The BMP antagonist Chd plays an important role downstream of organizer signals, but its depletion leads to only partially ventralized phenotypes, underscoring that multiple organizer genes are required for axial development. Depletion of *sia* with antisense morpholino oligonucleotides (MOs) affected mostly head and brain development, while reducing Nodal signaling with low doses of *cerS* inhibited mostly trunk-tail development (while at higher doses *cerS* inhibits all dorsal axis activity, ref. 29). Siamois may have originated in amphibians thorough the evolutionary recruitment of a mesodermal Nodal-inducible ancestor Mix-type homeobox gene related to *sebox* (skin-embryonic-brain-oocyte-specific homeobox) (37–39). We propose that Siamois became independent of Nodal signaling in *Xenopus* but retained many of the original Nodal-inducible organizer target genes, facilitating the rapid induction of the prospective neural plate. We compare the evolution of Siamois to that of zebrafish Bozozok, which derived through a different mechanism from the *goosecoid* homeobox gene, also becoming independent of Nodal signaling. Our main conclusion is that the *Xenopus* embryo has a double assurance mechanism ("belt and suspenders") by which Siamois or Nodal signaling independently and redundantly induces similar sets of organizer-specific gene targets to ensure axial development.

Results

β-Catenin Depletion Sensitizes *Xenopus* Embryos to Organizer Signals. To test the effect of β-catenin MO, in preliminary experiments, we injected embryos four times (from now on designated 4x β-cat MO) into the marginal zone of 2 to 4 cell embryos, which became ventralized with high penetrance (40). A complete axis can be readily induced by then injecting a single ventral blastomere (1xV) at the 4-to 8-cell stage with low doses of *chd* mRNA (29). Embryos receiving one-time dorsal (1xD) and one-time ventral (1xV) injections of 5 pg *chd* mRNA could induce two complete axes in the sensitized β-catenin-depleted background (Fig. 1 *A–D*). We next tested the effect of 1xV coinjection of *chd* mRNA and β-cat MO. In wild-type (WT) embryos *chd* mRNA induced only partial secondary axes lacking head structures even at 50 pg (Fig. 1*E*). However, when β-cat MO was included, secondary axes with large heads and brain structures with cement glands and two eyes were formed (Fig. 1 *F–H*). This result is concordant with the literature showing that double inhibition of BMP and Wnt signaling pathways by different means promotes complete head development in *Xenopus* (41–43). To investigate the cause of this striking cooperation, we coinjected ADMP MO together with *chd* mRNA. The organizer secretes ADMP, which provides a potent ventralizing BMP signal originating on the dorsal side (6, 7). ADMP MO coinjected with *chd* mRNA 1xV in WT embryos synergized to induce secondary axes with large brains and two eyes (Fig. 1 *I–L*).

We conclude from these initial experiments that β-cat MO provides a sensitized system for dorsal axis formation studies, likely in part by preventing ADMP expression in the organizer (7). This is congruent with reports in the literature indicating that ADMP inhibition promotes dorsal and notochord development (42–44). We next used β-catenin-depleted embryos to analyze Nieuwkoop and BCNE signals.

β-Catenin Is Required for the Induction of Dorsal Organizer Genes Through Two Distinct Mechanisms Involving Siamois and Nodal Expression. *Xenopus* dorsal–ventral (D–V) patterning has been the focus of intense investigations that have provided a saturation molecular screen of differentially expressed dorsal–ventral (D–V) genes (45, 46). In *Xenopus*, the maternal-zygotic transition is

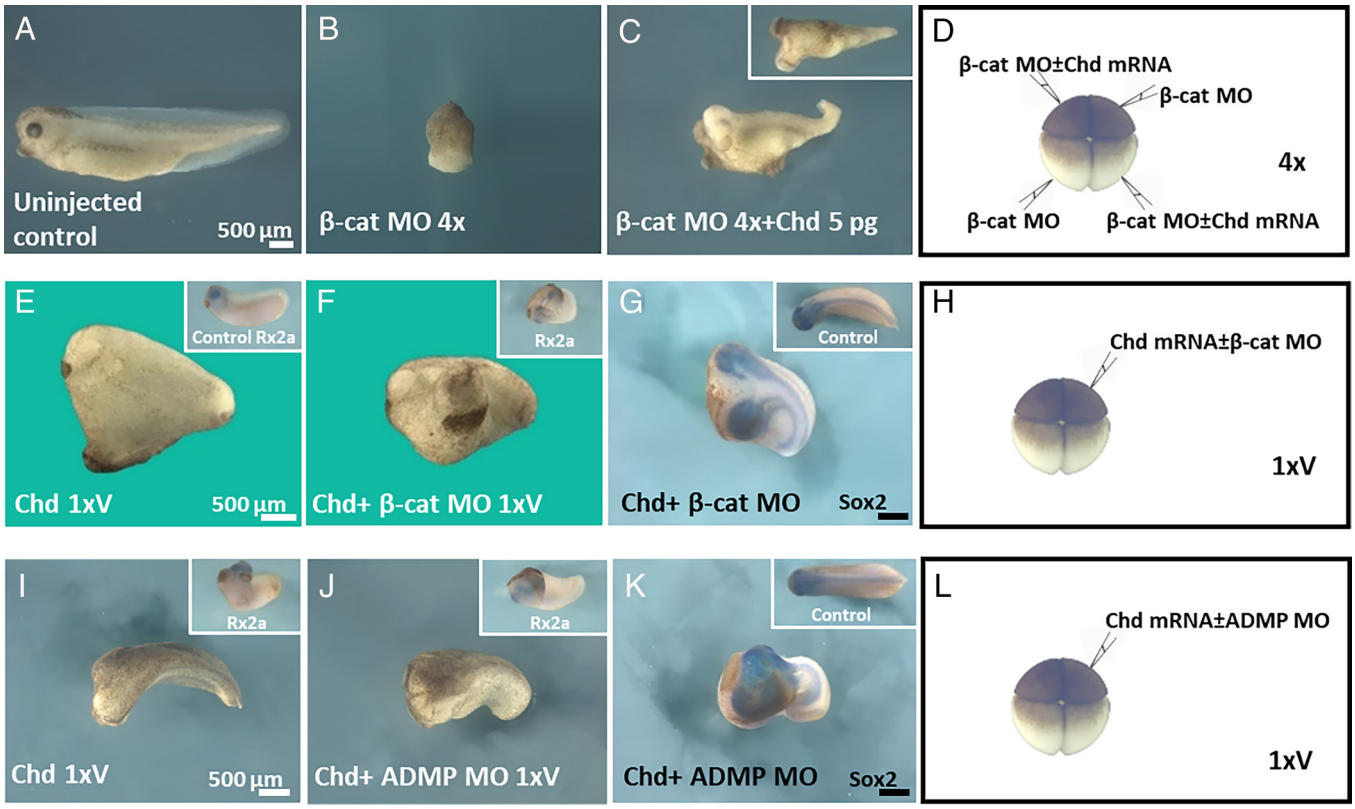
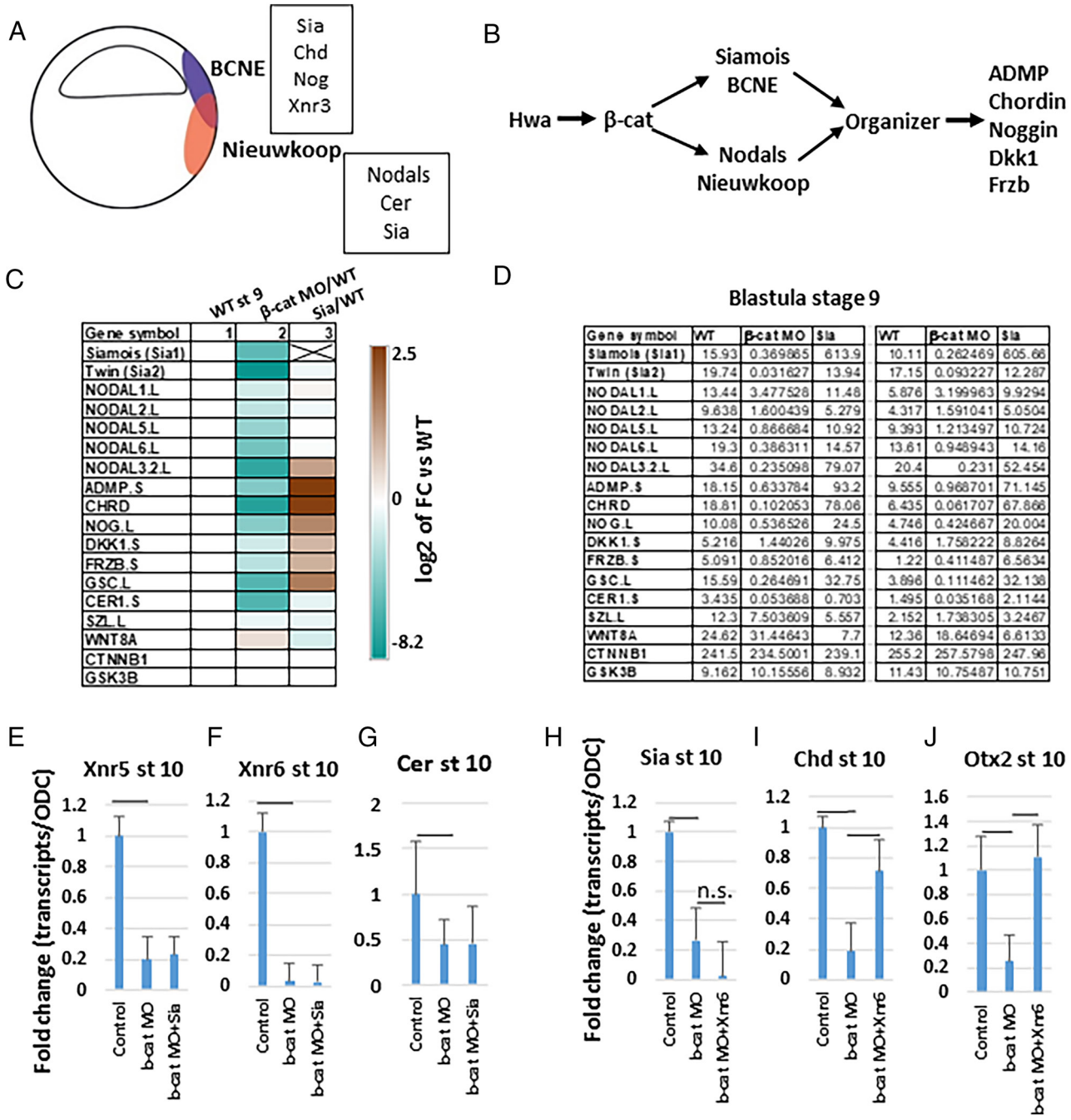


Fig. 1. β-catenin depletion provides a sensitized system to study axial development in *Xenopus*. (*A*) Uninjected control embryo. (*B*) β-catenin-depleted embryo with complete ventralization (*C*) Injection of *chd mRNA* 1 x ventral (1xV) plus 1 × dorsal (1xD) induced twin axes in β-catenin-depleted embryos. (*D*) Experimental design. (*E*) *chd mRNA* (5 pg) single injection into a ventral blastomere at the 4-cell stage caused incomplete second axis; inset, uninjected control embryo with eye marker *rx2a* staining only the primary axis. (*F*) Single ventral coinjection of *chd mRNA* and β-cat MO 1xV induced complete secondary axes; inset, the secondary axis stained with the eye marker *rx2a* showed two eyes. (*G*) Coinjection of *chd mRNA* (50 pg) and β-cat MO induced complete axes including brain structures stained with the pan-neural marker *sox2* (5 independent experiments); inset, control embryo stained with *sox2*. (*H*) Experimental design. (*I*) 1xV *chd mRNA* injected embryo with incomplete second axis; inset, *rx2a* staining showing that the incomplete secondary axis lacked eyes. (*J*) Single ventral injection of *chd mRNA* in combination with ADMP MO had strong twinned axis with duplicated cement glands (2 independent experiments); inset, positive *rx2a* staining in the secondary axis of *chd mRNA* plus ADMP MO. (*K*) 1xV *chd mRNA* injection together with ADMP MO showing the brain marked by *sox2* staining in the secondary head; inset, control uninjected embryo similarly stained. (*L*) Experimental design. The number of embryos was as follows: (*A*) n = 30, all normal; (*B*) n = 30, all completely ventralized; (*C*) n = 12, 10 with double axes; (*E*) n = 56, 46 with incomplete second axis and 10 with no second axis; (*F* and *G*), *chd* mRNA+β-cat MO 1xV, n = 147, 67 with complete second axis with cement gland, 67 with incomplete second axis stronger than *chd* alone mRNA phenotype, and 13 with very large cement gland in the primary axis or weak partial axes; (*I*) n = 41, 14 with partial darker second axis but without cement gland, 2 dorsalized, 1 dorsalized with large head and fused cement gland, 13 with weak axis, and 11 lacking second axes; and (*J* and *K*) n = 41, 32 with strong second axis with cement gland, 2 janus embryos consisting of two heads, and 5 incomplete second axis.

generally thought to take place at the 4000-cell midblastula stage 8 (47). However, even before midblastula transition (MBT) *xnr5* and *xnr6* are transcribed as early as the 256-cell stage (48). Similarly, *sia* genes start to be transcribed before MBT at stage 7 (33). Two signaling centers have been identified at blastula stage 9: the BCNE in the dorsal animal cap and the Nieuwkoop center in more vegetal cells, which overlap with each other in the marginal zone (18–20) (Fig. 2 *A* and *B*). We now made use of our genome-wide RNAseq data from Ding et al. (46) to examine the expression levels of the set of genes proposed recently to participate in the axial patterning pathway from egg to tailbud (29). When β-catenin depletion versus WT embryo expression levels were compared at blastula stage 9, *siamois*, *nodal1/2/5/6*, and organizer genes such as *chd*, *noggin*, *dkk1*, *frzb*, *gsc,* and *cerberus* (*cer*) displayed a strong requirement of β-catenin (Fig. 2 *C* and *D*). At stage 10.5, the expression levels of the organizer-specific zygotic genes were much higher (*SI Appendix*, Fig. S1). The expression of ventral genes such as *sizzled* (*szl*) and *wnt8* was not dependent on β-catenin. The D-V specificity in sibling embryo samples could be ascertained by comparing the transcriptomes of dorsal and ventral half-embryos dissected at stage 8 and allowed to regenerate until stage 10.5 (*SI Appendix*, Fig. S2; for the complete D-V transcriptome changes of 43,000 annotated *Xenopus laevis* genes see Dataset 1 from reference 46). The entire dorsal repertoire was induced by Lithium chloride (LiCl) treatment at the 32-cell stage, which stabilizes β-catenin through the inhibition of GSK3 (49, 50). Of note, β-catenin (CTTNB1) and GSK3β mRNAs were highly expressed at gastrula stages and not changed by any treatments or in dorsal/ventral fragments (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S1); in their case, phosphorylation control seems more important than mRNA levels.

Overexpression of *sia* mRNA 4x caused an increase in BCNE genes such as *chd*, *noggin*, *xnr3,* and *admp* but not of Nieuwkoop center markers such as *xnr5*, *xnr6,* and *cer* in RNAseq analyses (Fig. 2 *C* and *D*) at blastula. In β-catenin-depleted embryos, *sia* mRNA 1xV was unable to induce *xnr5, xnr6,* or *cer* by qRT-PCR at stage 10 (Fig. 2 *E–G*). In the converse experiment, microinjection of *xnr6* mRNA in β-catenin-depleted embryos did not induce the expression of *sia* although it increased organizer genes such as *chd* and *otx2* (Fig. 2 *H–J*). Thus, Sia did not induce *xnr5, xnr6,* or *cer*, and Xnr6 did not induce *sia*. These data indicate that the BCNE and Nieuwkoop centers can induce a related suite of organizer genes through distinct regulatory mechanisms: one mediated by Sia and the other by Xnrs (Fig. 3*A*).

We next tested whether 1xV microinjection of *sia* or *xnr6* mRNA into β-catenin-depleted embryos could rescue complete axial phenotypes. *sia* mRNA (10 pg) in WT embryos induced complete



Gene symbol	WT	β-cat MO	Sia	WT	β-cat MO	Sia
Siamois (Sia1)	15.93	0.369865	613.9	10.11	0.262469	605.66
Twin (Sia2)	19.74	0.031627	13.94	17.15	0.093227	12.287
NODAL1.L	13.44	3.477528	11.48	5.876	3.199963	9.9294
NODAL2.L	9.638	1.600439	5.279	4.317	1.591041	5.0504
NODAL5.L	13.24	0.866684	10.92	9.393	1.213497	10.724
NODAL6.L	19.3	0.386311	14.57	13.61	0.948943	14.16
NODAL3.2.L	34.6	0.235098	79.07	20.4	0.231	52.454
ADMP.S	18.15	0.633784	93.2	9.555	0.968701	71.145
CHRD	18.81	0.102053	78.06	6.435	0.061707	67.866
NOG.L	10.08	0.536526	24.5	4.746	0.424667	20.004
DKK1.S	5.216	1.44026	9.975	4.416	1.758222	8.8264
FRZB.S	5.091	0.852016	6.412	1.22	0.411487	6.5634
GSC.L	15.59	0.264691	32.75	3.896	0.111462	32.138
CER1.S	3.435	0.053688	0.703	1.495	0.035168	2.1144
SZL.L	12.3	7.503609	5.557	2.152	1.738305	3.2467
WNT8A	24.62	31.44643	7.7	12.36	18.64694	6.6133
CTNNB1	241.5	234.5001	239.1	255.2	257.5798	247.96
GSK3B	9.162	10.15556	8.932	11.43	10.75487	10.751

Fig. 2. Two independent signaling centers at blastula induce organizer-specific genes via the β-catenin targets Siamois and Xnrs. (*A*) Diagram of genes expressed at the blastula stage. The BCNE center expresses *siamois*, *chordin*, *noggin*, and *xnr3* whereas the Nieuwkoop center expresses *Xenopus* mesoderm-inducing *xnr1,2,5,6* and *cerberus*. (*B*) Scheme of the independent Siamois and Nodal pathways downstream of Hwa/β-catenin. (*C*) Heat map showing the log2 of fold change (FC) of early β-catenin-related genes at stage 9 WT controls, β-cat MO, and *siamois* mRNA injected embryos. Log2 of FCs over WT controls are indicated. (*D*) RPKM values determined by RNAseq at stage 9 in two WT control, two β-cat MO, and two *siamois* mRNA independent experiments. (*E–G*) *sia* mRNA was not able to induce *xnr5, xnr6,* or *cer*. (*H*) Conversely, *xnr6* mRNA did not increase *sia* expression in β-catenin-depleted embryos; both pathways are independent. (*I* and *J*) *xnr6* mRNA microinjection induced the *chordin* and *otx2* genes at stage 10 in β-catenin-depleted embryos by qRT-PCR. n.s. indicates not significant.

twinned axes, and in 4x β-cat MO background induced strong single axes with enlarged brain, eyes, and cement glands (Fig. 3 *B–I*). *xnr6* mRNA (1 pg) induced incomplete weak second axes in WT background (Fig. 3 *J, K, N*, and *O*) while at higher concentrations, it caused exogastrulation (in which the epiboly movements of the ectoderm and mesoderm fail to envelop the yolk endoderm). However, in β-catenin-depleted embryos, *xnr6* (1 pg) rescued complete dorsal axes (Fig. 3 *L, M, P*, and *R*). Coinjection of *sia* and *xnr6* mRNAs caused exogastrulation in β-catenin-depleted embryos (*SI Appendix* Fig. S3). Determining whether this strong phenotype is additive or synergistic would require curves of lower concentrations for each mRNA and their combinations, which is beyond the scope of this study. We conclude that, despite having different transcriptional activation mechanisms, Siamois and Xnr6 share the ability to induce complete dorsal axial development.

The Organizer Gene chd Plays an Important Role in Axial Induction by Huluwa, Siamois, and Xnr6. At blastula, Sia and Nodal signals cause the downstream activation of many Spemann–Mangold organizer genes, providing a double assurance mechanism for dorsal axis formation mediated by inhibition of ventral BMP signals (Fig. 4*A*). Chd is abundantly secreted by the organizer

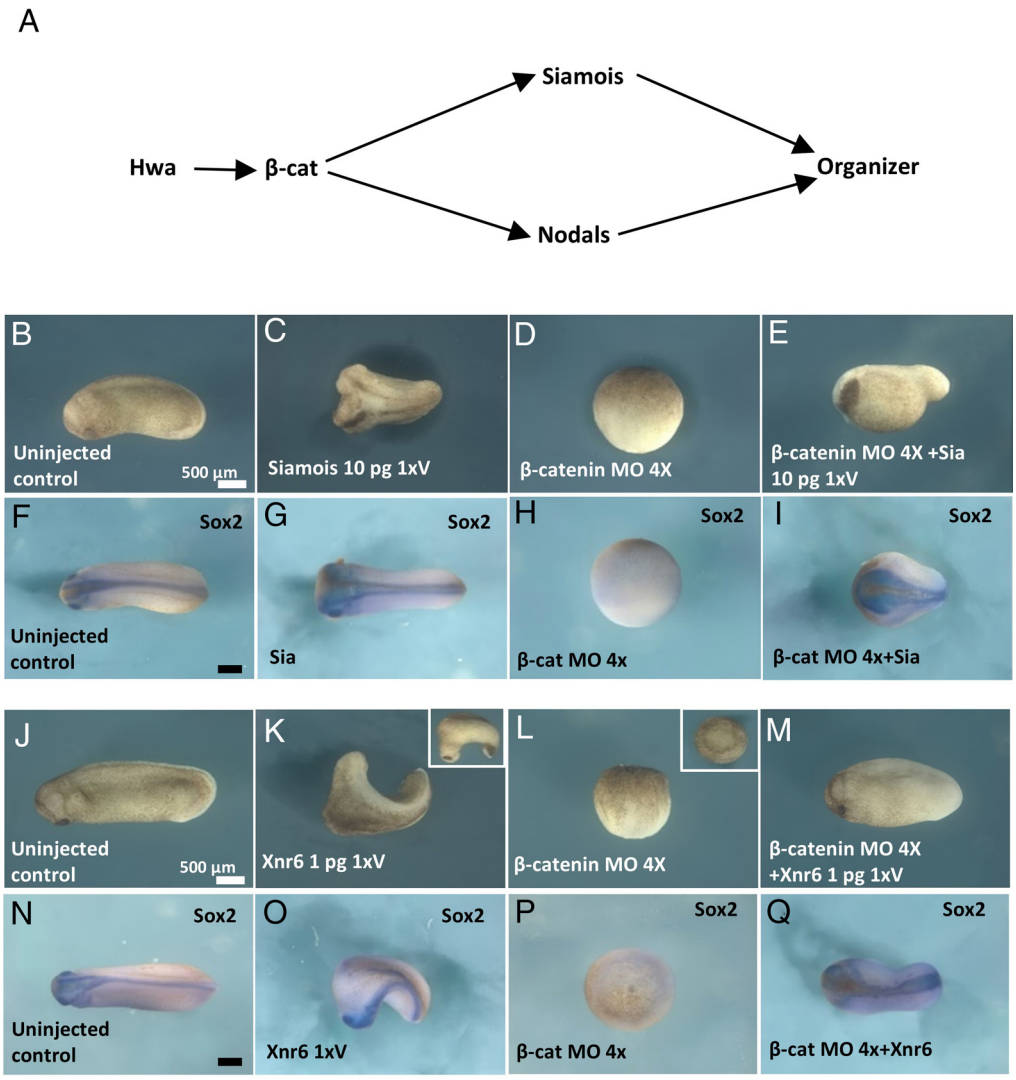


Fig. 3. Both Siamois and Xnr6 can rescue complete axes in β-catenin-depleted embryos. Embryos were injected with β-cat MO (40 ng) four times at the 2- to 4-cell stage and once ventrally with *siamois* (7.5 pg) or *xnr6* (1 pg) mRNAs. (*A*) Diagram of the Hwa/β-catenin/Siamois/Nodals pathways converging on the Spemann–Mangold organizer. (*B*) Uninjected control embryo. (*C*) 1 time ventral (1xV) *siamois* mRNA injected embryo with twinned axes. (*D*) Completely ventralized embryo by 4x β-cat MO injection. (*E*) *siamois* mRNA 1xV can rescue the primary axis in the β-catenin-depleted embryo; similar results were obtained in 4 independent experiments. (*F–I*) Pan-neural marker *sox2* staining for *B* to *E*. (*J*) Control embryo. (*K*) *xnr6* 1xV injected embryo with incomplete second axis. (*L*) Ventralized β-catenin-depleted embryo lacking axial structures. (*M*) Xnr6 was able to induce complete embryonic axes in β-catenin-depleted embryos, similar results were obtained in 3 independent experiments. (*N–Q*) pan-neural *sox2* staining for *J* to *M*, respectively. The number of embryos was as follows: (*B*) n = 70, all normal; (*C*) n = 102, 70 with second complete axis with cement gland, 17 with second incomplete axis, 13 dorsalized with enlarged cement gland, and 2 normal; (*D*) n = 60, 55 completely ventralized and 5 with short ventralized axes; (*E*) n = 121, 117 with induced axis with large cement gland, 2 weak axes, and 2 ventralized; (*J*) n = 47, all normal; (*K*) n = 83, 66 with incomplete second axes lacking cement gland with darker pigmentation, 9 normal, and 8 with dark spot near the anus; (*L*) n = 47, 45 with complete ventralization and 2 with partial ventralized axes; and (*M*) n = 54, all with complete axes.

(51). There are five *chd* genes in the *Xenopus laevis* genome, and a mixture of two well-characterized MOs has been previously found to inhibit Chd activity leading to partially ventralized embryos with small heads and expanded ventral–posterior tissues (52). To ensure more robust penetrance of the 4x Chd MO phenotype, we now added 2 more MOs targeting additional isoforms of *chd*. We microinjected 4x a mixture of these four Chd antisense MOs together with β-cat MO. The resulting embryos were entirely ventralized and indistinguishable from β-cat MO (Fig. 4 *D*, *Inset*). When Chordin and β-catenin-depleted embryos were injected 1xV with *sia* or *xnr6* mRNAs, the resulting embryos resembled the Chd MO depletion phenotype with small head and enlarged ventral tissues (Fig. 4 *B–I*). Thus, Chd is epistatic over the effects of Sia or Xnr6. However, a partial dorsal axis does still develop in Chd and β-catenin-depleted embryos; these axial structures may result from the activity of other organizer genes such as *noggin*, *follistatin,* and *dkk1*.

We next examined the requirement of Chd for signaling by the maternal cytoplasmic determinant Hwa (Fig. 5*A*). Depletion of Chd caused the typical small head phenotype (52) while 4x injection of *hwa* mRNA at 2.5 pg into WT resulted in complete radial dorsalization (Fig. 5 *B–D*). However, when *hwa* mRNA and Chd MOs were coinjected 4x, a single axis that phenocopied the Chd depletion phenotype was formed (Fig. 5*E*). The pan-neural marker *sox2* confirmed the reduction in the brain and eyes by Chd MOs even when Hwa was overexpressed 4x (Fig. 5 *F–I*). An interesting aspect of the Chd depletion phenotype is that it leads to strong expression of *sizzled* (*szl*) in the ventral–posterior mesoderm (51). *szl* is expressed at in regions with high BMP levels and is an inhibitor of the Tolloid proteinases that digest Chd and release active BMPs, such as ADMP, in ventral tissues (6, 51, 53), the *szl* expansion caused by Chd MOs was observed even in 4x *hwa*-injected embryos, indicating that Chd is necessary for the *hwa* mRNA overexpression phenotype (Fig. 5 *J–M*). To confirm that Chd was

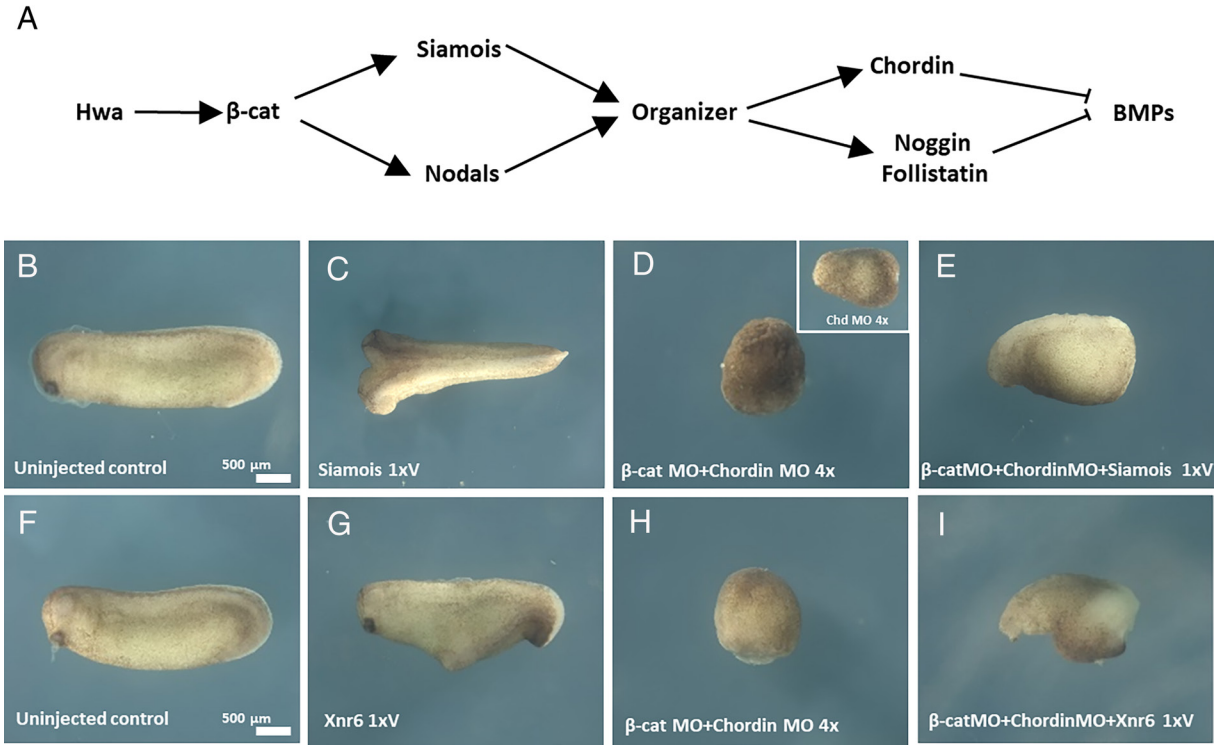


Fig. 4. Chordin was epistatic over the rescue of β-cat depletion by *siamois* or *xnr6* mRNA, yielding Chordin MO phenotypes. A mixture of 4 different Chd MOs (8 ng of each) was injected together with β-cat MO (8 ng) at 2 to 4 cells and rescued with *siamois* (7.5 pg) or *Xnr6* (1 pg) mRNAs by 1xV injection at 4 to 8 cells. (*A*) Diagram of Hwa/β-catenin/Sia/Nodals and downstream or Chd/Noggin/BMP pathway. (*B*) Control embryo. (*C*) *siamois* mRNA induced second axes. (*D*) β-cat MO plus Chd MOs 4x caused complete ventralization. (*E*) *siamois* can rescue β-catenin depletion but not the Chd MOs phenotype. (*F*) Uninjected control embryo. (*G*) Embryo with incomplete second axis induced by *xnr6* mRNA injection. (*H*) Completely ventralized embryo resulting from depletion of β-catenin and Chordin (*I*) *xnr6* mRNA was able to rescue β-catenin depletion but not the Chd MO phenotype with small head and expanded ventral tissue. The number of embryos, from three independent experiments, was as follows: (*B*) n = 87, all normal; (*C*) n = 24, 9 with complete second axis with cement glands and 15 dorsalized with enlarged cement glands; (*D*) n = 45, all ventralized; (*E*) n = 36, all had Chd MO phenotype with small heads and expanded ventral tissue; (*F*) n = 87, all normal; (*G*) n = 23, 2 with incomplete second axes with tail pigment, 17 with increased pigmentation in the proctodeum and 4 normal; (*H*) n = 44, all ventralized; and (*I*) n = 32, all with typical Chd depletion phenotype.

epistatic over *hwa* mRNA independently of the use of MOs, we used overexpression of *xolloid* mRNA, a secreted enzyme that degrades Chd (54). When *xolloid* was microinjected 4x at 250 pg, phenotypes very similar to those of Chd MOs were observed in 4x *hwa* embryos (*SI Appendix*, Fig S4). These results are remarkable in that *hwa*, which activates both the BCNE and Nieuwkoop centers by stabilizing β-catenin, has a complete requirement for the downstream target Chd for its ectopic overexpression phenotype.

Siamois Preferentially Affects Head Development, while Nodals Preferentially Regulate Trunk Development. Next, we asked whether Hwa signaling was affected differentially by *sia* or *nodals*. There are seven *sia* genes in *Xenopus laevis*, yet two specific MOs targeting Sia1 and Sia2 (Twin) have proven at least partially effective (55). When these MOs were coinjected 1xV with *hwa* mRNA at 10 pg, head structures in the second axis were preferentially inhibited (Fig. 6 *A–D* and *SI Appendix*, Fig. S5 *A–H*). *Xenopus laevis* also has multiple *xnr* genes and they all can be inhibited by *cerS*. We have previously reported that *hwa* mRNA coinjected with *cerS* mRNA at 500 pg loses second axis induction activity completely (29). However, we now report that at lower doses (50 to 150 pg) *cerS* tends to inhibit preferentially trunk structures, leaving some head-like structures (Fig. 6 *E–H* and *SI Appendix*, Fig. S5 *I–L*); this is probably explained by residual Sia activity in the Nieuwkoop center. 1xV *cerS* mRNA at 150 pg had no effect or a slightly ventralizing effect on its own (Fig. 6 *E* and *F*), but in combination with *hwa* mRNA it resulted in head-like phenotypes without trunk, reminiscent of full-length of Cerberus (56) (Fig. 6*H*).

As indicated in Fig. 6*I*, the broad stabilization of β-catenin on the dorsal side initiated by Hwa results in the formation of partially overlapping signaling centers at blastula under differential regulation by Sia and Xnr. Intriguingly, both signals are funneled through the same downstream target genes to form the dorsal axis.

Discussion

Redundant Signaling by Siamois and Nodals in *Xenopus*. We have recently proposed a dorsal axis molecular pathway starting with Hwa in the egg that stabilizes β-catenin that through Nodal-related signals at blastula activates the gastrula organizer pathway (29). This linear pathway was based on the observation that in a sensitized system provided by β-catenin-depleted embryos, *xnr1* or *xnr6* mRNAs induce a complete well-patterned axis from head to tailbud. This raised the question of what is the role of *sia*, a well-studied homeobox gene (29) in the pathway of *Xenopus* axis formation, providing the starting point for the present investigation. We found that *sia* mRNA was able to rescue complete axes downstream of β-catenin. This activity of *sia* and *xnr6* mRNAs required the function of the BMP antagonist Chd, since when Chd was depleted the resulting embryos had small brains and expanded ventral tissues in the typical Chd-deficient phenotype (51, 52). The organizer expresses many other BMP and Wnt antagonists that are presumably responsible for the residual dorsal axis activity. In addition, the dorsalizing phenotypes caused by overexpression Huluwa 4x in WT embryos showed a complete requirement for Chd. In view of these results, we have modified the proposed pathway (29) to indicate that Sia and Nodals provide

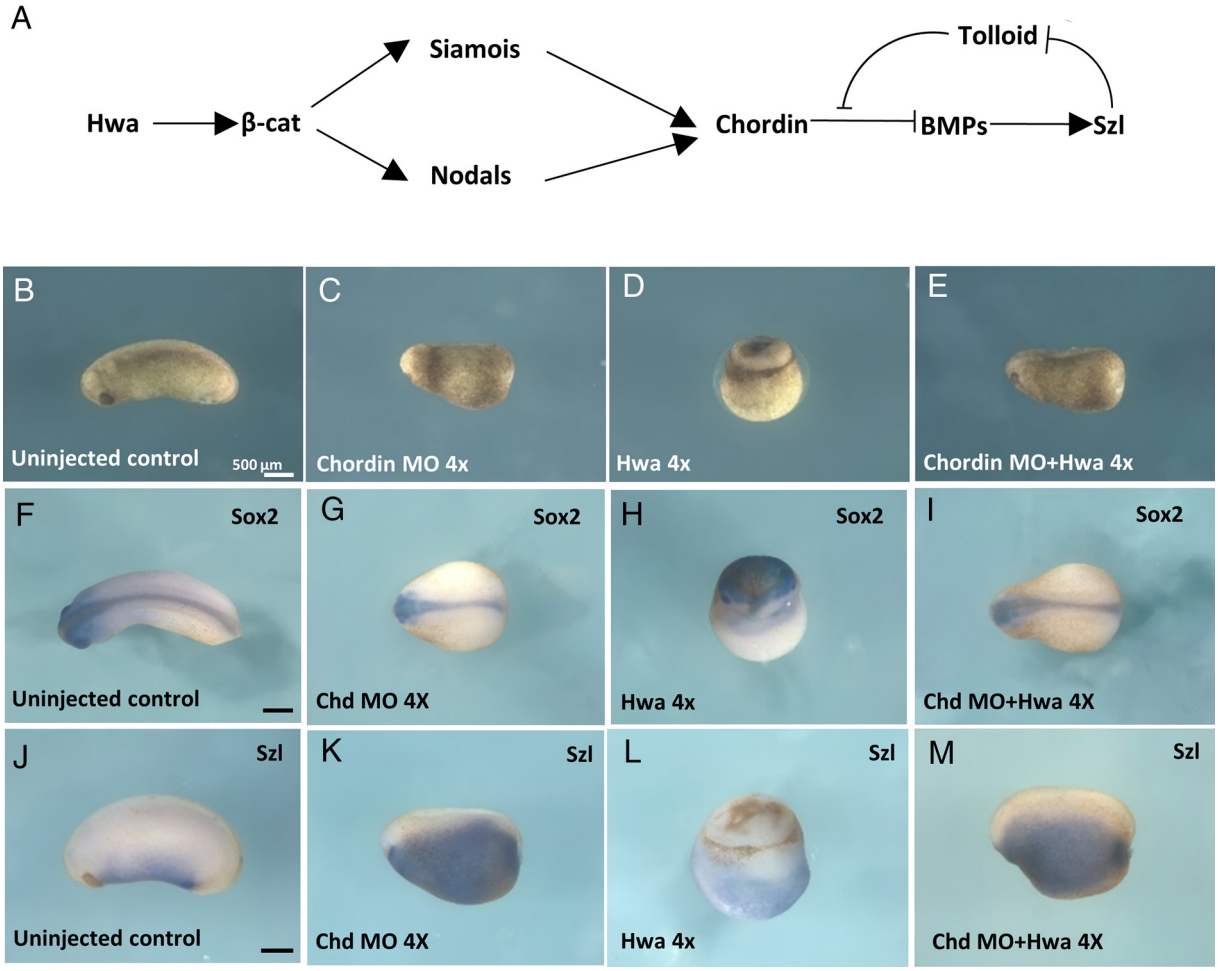


Fig. 5. Radial dorsalization by 4x *hwa* required Chordin. Embryos were injected at the 4-cell stage into each blastomere with 2.5 pg *hwa* mRNA together with 4 Chd MOs. (*A*) Diagram of the Hwa/β-catenin/Sia/Nodals/Chd/BMP/Szl/Tolloid signaling pathway. (*B*) Control sibling. (*C*) 4x Chordin depletion caused the typical partial ventralization. (*D*) Radially dorsalized embryo by 4 x injection of *hwa* mRNA. (*E*) Chordin was epistatic over the ectopic effects of *hwa* 4x. (*F–I*) *sox2* and (*J–M*) *szl in situ* hybridizations of *B* to *E* samples; note the reduction of the CNS and expansion of Szl in the ventral indicating elevated BMP signaling. The number of embryos was as follows: (*B*) n = 30, all normal; (*C*) n = 11, all partially ventralized with small head; (*D*) n = 22, 4 radially symmetic heads, 1 janus, 4 double axes with dorsalized phenotype, 1 double axis, and 12 strongly dorsalized with reduced trunks; and (*E*) n = 17, all had the Chordin depletion phenotype.

redundant signals upstream of mesodermal Spemann–Mangold organizer genes (*SI Appendix*, Fig S6).

Two Independently Regulated Signaling Centers in the *Xenopus* Blastula. Embryos devoid of the mesoderm by injection of the specific Nodal inhibitor *cerS* (or of the Nodal/Activin inhibitor Lefty) lack axis formation yet form a brain with an eye structure (19). Using lineage tracing, this activity could be traced at blastula to the expression of *sia*, *chd*, and *noggin* downstream of β-catenin (18). This region is fated to form the brain and floor plate in WT embryos and was designated the BCNE center. The dorsal animal cap of the blastula has a predisposition toward neural induction when compared to the ventral ectoderm (57, 58), and cultured sandwiches of dorsal ectoderm explants develop into CNS tissue (18). In *Xenopus laevis*, there are seven *sia* genes (and four in *Xenopus tropicalis*), and their expression has been studied in detail (33). Transcription of *sia* starts before midblastula transition and is initially found in the dorsal animal cap but is also found, particularly at gastrula stages, in the marginal region (33). The BCNE center is more of a functional definition than a spatial one, for *sia* expression is independent of Nodal and mesoderm formation yet overlaps partially with the Nieuwkoop signaling center driven by early Xnr5 and Xnr6 in more vegetal regions (19, 20).

The dorsal Nieuwkoop center is responsible for the induction of the organizer in the overlaying mesoderm through Nodal inductive signals (13, 17). The body axis of chordates is dependent on Nodal signaling, which provides essential axial inductive signals in mouse and chick (59, 60). In *Xenopus*, the earliest Nodals expressed are Xnr5 and Xnr6 in the dorsal endoderm (48, 61). In embryos from *Amphioxus* to humans, Nodal signals are required for primitive streak, hypoblast, and anterior visceral endoderm specification to establish the anterior–posterior axis (62–64).

Genetic screens in zebrafish have contributed much of our current understanding of gastrulation mechanisms. The Nodal genes *squint* and *cyclops* play a fundamental role in axial development (65, 66). The morphogen Squint is expressed downstream of β-catenin in the dorsal yolk syncytial layer (YSL) that would be homologous to the amphibian Nieuwkoop center. A second gene expressed in the dorsal YSL was identified by the *bozozok* mutant, which encodes a homeodomain protein also essential for dorsal development (67). Bozozok acts downstream of β-catenin and is independent of Squint, and in double *bozozok;squint* mutants, the anterior CNS and axial mesoderm are lost (68). Bozozok directly represses *bmp2* transcription (69, 70). Bozozok also strongly cooperates with Chordin and in double *bozozok;chordino* mutants, head and trunk axial structures are lost, with tail structures remaining (71). Vertebrates require

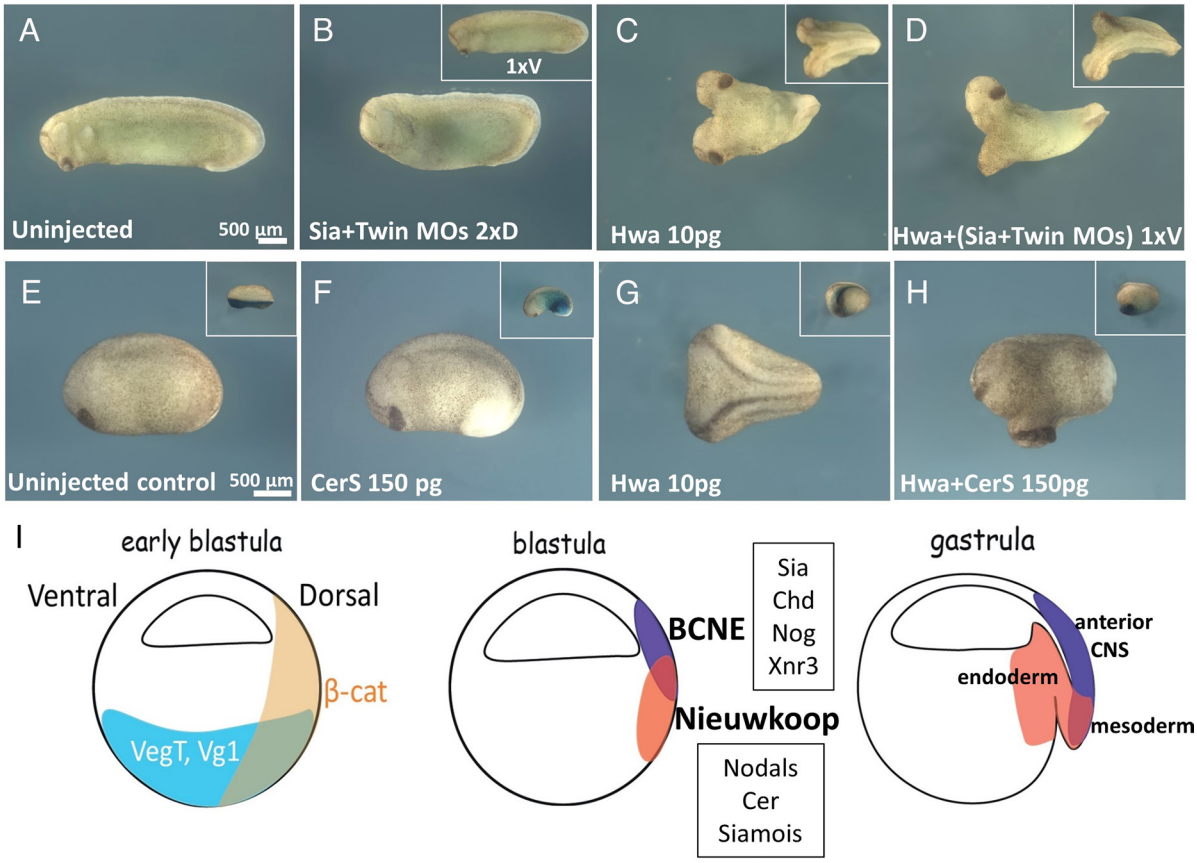


Fig. 6. Depletion of Siamois and Twin with specific MOs interfered with head development while the Nodal antagonist Cerberus-short (CerS) inhibited preferentially trunk development. (*A*) Control uninjected embryo. (*B*) Sia plus Twin MOs two times dorsal injection partially reduced head structures; inset, a single ventral injection of Sia/*Twin* MOs was without phenotypic effect. (*C*) *hwa* mRNA (10 pg) 1xV induced complete twinned axes. (*D*) 1xV injection of *hwa* together with Sia/Twin MOs caused defects in head and cement gland formation while the trunk was less affected. (*E*) Uninjected control embryo. (*F*) Single injection of 150 pg *Cerberus-short* mRNA into a ventral blastomere. (*G*) Embryo with complete second axis induced by *hwa* 10 pg. (*H*) Coinjection of *hwa* and *cerS* mRNAs decreased trunk formation but preserved head-like structures at this concentration. Insets in *E*–*H* show the fate of microinjected cells using *lacZ* mRNA lineage tracer. (*I*) Diagram of early embryonic signals at cleavage/early blastula, blastula, and gastrula stages; the BCNE is driven by Siamois and the Nieuwkoop center by mesoderm-inducing Nodals. The diagram was drawn using Adobe illustrator. The number of embryos, from 3 independent experiments, was as follows: (*A*) n = 92, all normal; (*B*) n = 39, 28 ventralized with reduced cement gland, 1 ventralized with no cement gland, and 10 normal; inset n = 44, all normal; (*C*) n = 78, 73 with a double complete axis and 5 with a second induced axis contains smaller cement gland compared to the primary axis; (*D*) n = 92, 49 with incomplete second axis with lacking cement glands, 17 with small cement glands in the 2nd axis, 2 with lateral bumps, 20 with complete 2nd axes, 2 dorsalized, and 2 unaffected; (*E*) n = 46, 1 partially ventralized; (*F*) n = 46, slight ventralization was noted; (*G*) n = 77, 68 with complete twinned axes, 6 incomplete second axis, and 3 unaffected; and (*H*) n = 121, 94 with second ectopic head-like structures lacking trunks, 15 bumps but no distinctive ectopic head structures, and 12 with no secondary axes.

Nodal/TGF-β signals for organizer induction, but in addition, frogs and fish have homeobox genes downstream of β-catenin that are activated independently of Nodals. Although superficially regulation appears similar in fish and frogs, we will discuss below how they likely have quite different evolutionary origins.

Double Assurance in Embryonic Induction Facilitates Neural Induction. The phenomenon of embryonic induction was first discovered in 1901 with the induction of crystalline lens by neural eye vesicles on the overlying ectoderm and subsequent work showing that the surface ectoderm also played a role (72). This reciprocal interaction received the name of double assurance (*doppelte Sicherung*, a principle derived from engineering) more colloquially designated as "belt and suspenders" in development. The induction of the neural plate by the underlying endomesoderm, considered the primary embryonic induction, has attracted the most attention by experimental embryologists. Spemann dedicated Chapter 8 of his book to the problem of whether the ectoderm plays a role in neural plate induction by organizer (72). Some early experiments in which the ectoderm was damaged suggested that the ectoderm might be involved (73). However, this proposal came to an end with the famous exogastrulation experiment of Holtfreter in which the axolotl ectoderm evaginated from the endoderm differentiated into pure epidermis (74, 75). Recently, the double assurance problem was revisited, and it was found not only that the BCNE gives rise to the future brain and floor plate but also that expression of Chordin and Noggin in these cells is required for brain induction (18). The BCNE center, which is driven by Siamois in the animal cap, has no inductive activity when transplanted into other regions of the embryo and thus its developmental importance went unnoticed, with attention focused on signals emanating from the Spemann–Mangold organizer of the gastrula endomesoderm (18, 52). Interestingly, use of a hormone-inducible version of Sia showed that the critical period for Sia function is during blastula and is lost 2 h before the start of gastrulation, coinciding with its expression in the BCNE center (76). Both *sia* and *xnr5/6* are primary response genes to β-catenin, and their redundant function downstream of this signal can be explained by a double assurance mechanism provided by the BCNE and Nieuwkoop centers at blastula stages.

Two Different Molecular Mechanisms Have Recruited a Common Set of Organizer Genes. An interesting question is how the same cocktail of organizer-specific genes has been recruited to respond to Nodals or Siamois in *Xenopus*, and Squint or Bozozok in zebrafish. The early Nodal/TGF-β signal, via the phosphorylation of the

Fig. 7. Sequence alignments of the homeodomains of Siamois, Sebox, Bozozok, and Goosecoid. (*A*) BLAST analysis revealed that the 60-amino acid homeodomain of Siamois was most closely related to that of Sebox (31/60), a homeodomain gene belonging to the Mix family of transcription factors. Identical amino acids are highlighted in blue. (*B*) Sequence alignment between the *Xenopus* transcriptional activator Siamois and the zebrafish transcriptional repressor Bozozok showing that their homeodomains are very divergent (23/60); the critical amino acid at position 50 that determines DNA sequence recognition specificity (arrowhead) changed from Q to K, which is a characteristic of the Bicoid and Goosecoid homeodomains. (*C*) Sequence comparison of zebrafish Bozozok and Goosecoid homeodomains revealed that they share the highest degree of amino acid identity (34/60) among all homeodomain proteins.

Smad2/3 transcription factors, is essential in mammals and crucial for axis formation (70, 77). The requirement for the homeobox β-catenin primary response genes *sia* and *bozozok* might correspond to specialized evolutionary adaptations for rapid development.

Siamois encodes a homeodomain of the Paired superfamily (78). In blast searches with the 60 amino acids of homeodomain of Siamois is closest to that of Sebox (skin-embryonic-brain-oocyte-specific homeobox), a gene isolated by the Nirenberg lab in humans and the mouse (36). (Fig. 7*A*). *Xenopus* also has a *sebox* gene, which is part of the Mix/Bix homeodomain family (79). The founder *Mix* gene was discovered as a *Xenopus* primary response gene to Activin signaling (80). Other genes in this family are the Bix1-4 (Brachyury-induced homeobox) and Mixer, all of which are activated by Nodal signals in the endomesoderm of *Xenopus*. In mammals, in addition to Sebox, this family is represented by a single Mixl1 (Mixer-related 1) required for mesendoderm development (81). *Xenopus* Sebox has been studied in detail, and its expression starts on the dorsal side at early gastrula, from where it then expands to the entire marginal zone and is required for mesoderm formation (39). Since *sia* appears to have evolved from a *sebox-like* homeobox gene, it is possible that its original function was to activate dorsal mesoderm genes, including the organizer repertoire downstream of β-catenin and Nodal. As Siamois became specialized, its protein may have become able to induce transcription without requiring the cooperation of the Nodal/Smad2/3 signal while retaining its original organizer target gene set. The scenario above is of course speculative, and it is also possible that many organizer gene enhancers acquired Siamois binding sides independently multiple times in evolution.

What about Bozozok? Sequence alignments between *Xenopus* Siamois and zebrafish Bozozok show that the homeodomains are highly divergent. The amino acid at position 50 in Goosecoid, which determines DNA sequence recognition has been changed from Glutamine (Q) to Lysine (K) (Fig. 7*B*, arrowhead). Lysine in position 50 is found in *Drosophila* Bicoid and *Xenopus* Goosecoid (78). As originally described, and confirmed by our recent blast searches, the homeodomain of Bozozok (a protein also known as Dharma) is most related to *goosecoid* (Fig. 7*C*) (82). *goosecoid* is an ancient homeobox gene present in *Drosophila* and all vertebrates (83). Since Bozozok appears to have evolved from a *goosecoid*-like gene, its origin was very different that of Siamois/Sebox, yet both regulate the organizer target gene repertoire. Like Goosecoid, Bozozok is a repressor transcription factor while Siamois is an activator (35, 69). Expression of *goosecoid* in the gastrula mesoderm requires Nodal signaling (84), yet its relative Bozozok is independent of Nodal and is activated in parallel by the early β-catenin signal. Unlike Siamois, Bozozok is not expressed in the prospective ectoderm but rather in deep tissues of the YSL and endoderm (82). However, the transcriptional activation of Bozozok, like Siamois, lacks the requirement for Nodal/Smad2/3 signals. These considerations suggest that despite both genes cooperating with Nodal/Smad2/3 signals, the Siamois and Bozozok homeodomain genes are specializations of amphibians and fish embryos that have distinct evolutionary origins and mechanisms. While some homeodomains such as those of the Hox complexes are highly conserved, others can diverge rapidly (78), as was the case for Siamois and Bozozok.

The early stages of development have been subjected to many evolutionary modifications due to different requirements for gastrulation mechanisms. Some steps of the dorsal axis pathway are invariant, such as early dorsal β-catenin, Nodals, and BMP and Wnt antagonists needed to generate an embryonic region devoid of BMP signaling through which dorsal fates can be differentiated. Other steps are variable in different vertebrate groups. For example, the *Xenopus* blastula BCNE driven by Siamois is likely an adaptation to facilitate the rapid induction of the anterior neural plate by underlying Spemann–Mangold organizer signals (Fig. 6*I*). The need for rapid development in a dangerous pond water environment resulted in duplications of many gastrulation genes. There are seven *siamois* genes in *Xenopus laevis* resulting from tandem duplications, eleven *xnr* genes including at least three tandem duplications of *xnr5*, and five *chd* genes in *Xenopus laevis* (33). During evolution, ancient genes can also be lost. For example, ADMP and Sizzled, which play fundamental roles in signaling by the organizer in fish, amphibians, and birds, have been lost in mammals, perhaps due to the loss of yolk in the egg (85). In addition, Hwa provides the initial maternal signal upstream of β-catenin in zebrafish, frog, echinoderms, and reptiles but has been lost in amniotes (21). Despite the existence of evolutionary adaptations, the core axial patterning system established in zebrafish and *Xenopus* remains paradigmatic.

Materials and Methods

***Xenopus* Ethic Statements.** *Xenopus* experiments were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the NIH. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California Los Angeles (Medical

Table 1. Forward and reverse primer sequences for qRT-PCR

Gene	Forward primer	Reverse primer
odc	CAGCTAGCTGTGGTGTGG	CAACATGGAAACTCACACC
chordin	CCTCCAATCCAAGACTCCAGCAG	GGAGGAGGAGGAGCTTTGGGACAAG
siamois	AAGATAACTGGCATTCCTGAGC	GGTAGGGCTGTGTATTTGAAGG
xnr6	AGATTGAGGAAAGTACCAGTTT	GCTTTCCATTATCCTTTGGC
otx2	GGATGGATTTGTTACATCCGTC	CACTCTCCGAGCTCACTTCCC
admp	GATGATGGAAGGAGAGGA	TCATGTTCTGACCCAAAG

School – Permit Number: ARC-1995-129). Mature pigmented females and male albino *Xenopus laevis* were purchased from *Xenopus*-1 Inc. (Dexter, MI).

***Xenopus* Embryo Manipulations and Whole Mount In Situ Hybridization.** Unfertilized eggs were placed in 100 x 20 mm plastic dishes (Fisher FB0875711Z) with minimal liquid and fertilized in vitro with freshly dissected testis as described (29). The mixture was incubated for 5 min at room temperature (RT). Later, 0.1x MMR (Marc's modified Ringers) was added, and the dishes were left for 70 min at RT. Fertilized embryos were dejellied with freshly prepared 2% cysteine solution in 0.1x MMR (pH 8.0) for 10 min at RT, followed by three washes with 0.1x MMR. Embryos were microinjected in 1x MMR in 60 x 15 mm dishes (Fisher FB0875713A) coated with 2% agar (Fisher, BP1424-500) in H_2O, then transferred to 0.1x MMR agar dishes. Next, embryos were incubated at 15 °C overnight to improve survival. Embryos were fixed in 0.5x MEMFA (0.05 M MOPS pH 7.4, 1 mM EGTA, 0.5 mM MgSO4, and 3.7% formaldehyde) in 1 Dram vials (Fisher 03-339-25B).

mRNA Synthesis and Morpholinos. pCS2-*chordin*, pCS2-*siamois*, pCS2-*Xnr6*, pCS2-*xolloid*, pCS2-*cerberus-short,* and pCS2-*nlacZ* were linearized with NotI and pCS107-*Xhwa* was digested for linearization with Hpa1. Next, mRNAs were synthesized with the mMessage mMachine SP6 kit, including SP6 RNA polymerase (Invitrogen, Massachusetts). They were purified using the MegaClear kit (Invitrogen, Massachusetts). mRNAs were injected 1 time ventral or 4 times into blastomere at the 4- or 8-cell stage at the indicated amounts in a volume of 4 nl. *Xenopus laevis* antisense β-cat MO (5′ TTTCAACCGTTTCCAAAGAACCAGG 3′), ADMP MO (5′ GGTCCATCTCATCAAGCTGCAGCTC 3′), and a mixture of four Chd MOs (1st, 5′ ACGTTCTCTCTCGTATAGTGAGCGT 3′; 2nd, 5′ ACAGCATTTTTGTGGTTGTCCCGAA 3′; 3rd, 5′ GGAACACAGCATTTTTGTGGTAGTC 3′; 4th, 5′ GGGACACTGCATTTTTGTGGTTCCA 3′) were purchased from Gene Tools, LLC (Philomath, OR, USA). 10 ng of β-cat MO was injected four times radially (total 40 ng) at the 2- to 4-cell stage. 10 ng of ADMP and cocktail of 4 Chd MOs were injected into each blastomere (total 40 ng) at the 2- to 4-cell stage.

LacZ Staining and In Situ Hybridization. Embryos were fixed in 0.5x MEMFA for 1 h, followed by three 10-min washes with PBS (Fisher Scientific, BP3994) and incubated overnight at 4 °C in X-Gal staining solution (1 mM X-Gal, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$ in PBS). After washing twice with PBS for 5 min each, refixation was done in 0.5x MEMFA for 2 h at room temperature. In situ hybridizations were performed following the protocol detailed in the De Robertis Lab website (https://www.hhmi.ucla.edu/derobertis). Embryos were imaged using a ZEISS Axio Zoom.V16 dissecting microscope with Z-stack function for capturing three-dimensional information.

qRT-PCR. Quantitative real-time reverse transcription PCR (qRT-PCR) analyses were performed at stage 10, following a previously established method (86). Primer sequences for qRT-PCR are given in Table 1.

Heatmap and RNAseq Analysis. The RNAseq data previously published by Ding et al. (46) (Dataset 1 and Dataset 7) were used in this paper. Organizer genes were selected from the 43,000 genes in the Excel file. The average expression values were divided by the average expression in Control embryos, and the log2 of these ratios used in the heatmaps. The WT/β-catenin and Siamois/Control ratios used stage 9 embryo samples, and the log2 of WT/β-catenin, Siamois/Control, and LiCl/WT ratios were employed at stage 10.5. To identify genes with the highest fold change (FC), various conditions/WT ratios were sorted from highest to lowest in Excel. Some genes were annotated in the initial original *X. laevis* JGI 9.1 genome simply as *Xlaev* ID numbers (e.g., *siamois*, *chordin*). To identify their matches in the more advanced JGI 10.1 genome assembly, we used the *Xlaev* JGI 9.1 numbers to identify the corresponding XM IDs in NCBI. This sequence was then used in NCBI nucleotide BLAST to identify gene correspondences.

Homeodomain Alignments. For the alignment of *sia*/*sebox*, *sia*/*boz*, and *boz*/*gsc*, the UniProt website was used. The following proteins were found in UniProt: *Xenopus* siamois (Q91848, SIAM_XENLA), *Xenopus* sebox.L (A0A974HXQ4, A0A974HXQ4_XENLA), zebrafish bozozok/dharma (O93236, O93236_DANRE), and zebrafish goosecoid (P53544, GSC_DANRE). The homeodomains of these proteins, consisting of 60 amino acids, were aligned.

Statistical Analyses. Data were expressed as means using SD of the means. The Student *t* test was calculated in Excel and was used for statistical analysis. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ between the means of each sample were considered as statistically significant.

Data, Materials, and Software Availability. All results are present in the article and *SI Appendix*. The complete datasets of RNAseq used here have been published previously (46) and are available at the Gene Expression Omnibus (GEO) database with the accession number GSE93195. No new software was generated in the present study.

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