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Molecular analysis of a self-organizing signaling pathway for *Xenopus* axial patterning from egg to tailbud

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Xenopus embryos provide a favorable material to dissect the sequential steps that lead to dorsal–ventral (D-V) and anterior–posterior (A-P) cell differentiation. Here, we analyze the signaling pathways involved in this process using loss-of-function and gain-of-function approaches. The initial step was provided by Hwa, a transmembrane protein that robustly activates early β-catenin signaling when microinjected into the ventral side of the embryo leading to complete twinned axes. The following step was the activation of _Xenopus_ Nodal-related growth factors, which could rescue the depletion of β-catenin and were themselves blocked by the extracellular Nodal antagonists Cerberus-Short and Lefty. During gastrulation, the Spemann–Mangold organizer secretes a cocktail of growth factor antagonists, of which the BMP antagonists Chordin and Noggin could rescue simultaneously D-V and A-P tissues in β-catenin-depleted embryos. Surprisingly, this rescue occurred in the absence of any β-catenin transcriptional activity as measured by β-catenin activated Luciferase reporters. The Wnt antagonist Dickkopf (Dkk1) strongly synergized with the early Hwa signal by inhibiting late Wnt signals. Depletion of Sizzled (Szl), an antagonist of the Tolloid chordinase, was epistatic over the Hwa and Dkk1 synergy. BMP4 mRNA injection blocked Hwa-induced ectopic axes, and Dkk1 inhibited BMP signaling late, but not early, during gastrulation. Several unexpected findings were made, e.g., well-patterned complete embryonic axes are induced by Chordin or Nodal in β-catenin knockdown embryos, dorsalization by Lithium chloride (LiCl) is mediated by Nodals, Dkk1 exerts its anteriorizing and dorsalizing effects by regulating late BMP signaling, and the Dkk1 phenotype requires Szl.

Huluwa | β-catenin | Cerberus | Chordin | Dickkopf 1

Significance

Xenopus embryos provide a model system to dissect the sequential steps that lead to the development of the vertebrate body axis. The first event is the function of the maternal dorsal determinant Huluwa, a new protein discovered in China in 2018, that stabilizes cytoplasmic β-catenin by promoting Axin1 degradation. We combined overexpression of *Hwa* mRNA into a ventral cell with coinjection of known components of the Spemann–Mangold organizer biochemical pathway. We found that, unexpectedly, well-patterned complete embryonic axes are induced by Chordin or Nodal in β-catenin-depleted embryos, that dorsalization by Lithium chloride (LiCl) is mediated by Nodals, and that the Wnt antagonist Dickkopf 1 strongly synergizes with Huluwa downstream of BMP signaling at a later stage of development.

Embryonic development from egg to tailbud is a fascinating process. In 1895 Morgan demonstrated that removing one of the two blastomeres in a frog embryo could result in the formation of a perfect half-embryo, and in 1903 Hans Spemann showed that constricting newt embryos with baby hair could induce the formation of twins (1). Further, recent studies using *Xenopus laevis* as a model organism revealed that a blastula embryo cut in half can generate identical twins, provided both halves contain dorsal components (2). Thus, the embryo has a tendency to self-organization, and the cell signaling mechanisms involved in this process are still incompletely understood despite intense studies.

The most fundamental discovery in this field took place a century ago when Spemann and Hilde Mangold demonstrated the powerful inductive activity of the dorsal lip of the blastopore, the site where endo-mesoderm invagination begins. This region induces the formation of the central nervous system (CNS) and dorsal mesoderm structures such as somite and kidney (3). This pioneering experiment was the basis for our current understanding of vertebrate development as a series of cell–cell inductive interactions, and its centennial is being celebrated this year (4). With the advent of molecular cloning, many genes encoding proteins secreted by the organizer were isolated. These include Bone Morphogenetic Protein (BMP) antagonists such as Chordin and Noggin, Wnt antagonists such as Dkk1, Frzb-1, and others, Cerberus (an inhibitor of Nodal, Wnt, and BMP), Lefty/Antivin (an inhibitor of Nodal and Activin), and Anti-Dorsalizing Morphogenetic Protein (ADMP, a BMP produced in the Organizer) (5). Studies on the Spemann–Mangold gastrula organizer have revealed many novel molecular signaling mechanisms. For example, embryonic patterning by Chordin is regulated through its proteolysis by Tolloid proteinases, which in turn can be inhibited by Sizzled (Szl), a protein secreted by the ventral center of the gastrula (6–8).

The organizer, established at the gastrula stage, is capable of inducing head, trunk, and tail structures. However, the process of axis formation is a continuum that starts at fertilization and continues into the early tailbud stage. Mesoderm induction by endoderm takes place at the blastula stage (9). Experiments recombining endoderm explants with animal cap ectoderm (10, 11) suggest that *Xenopus* Nodal-related factors (Xnrs) induce both dorsal

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and ventral mesoderm. Low Nodal concentrations induce ventral mesoderm, while high concentrations lead to the formation of dorsal mesoderm (Spemann organizer) in the overlaying ectoderm (11, 12). Nodal is a Transforming Growth Factor β (TGF-β) discovered as a mutation required for mouse gastrulation (13).

An important advance in understanding the initiation of the Spemann–Mangold organizer pathway was the discovery of a zebrafish maternal mutation that completely lacks the dorsal axis by the laboratory Anming Meng (14). It was named *Huluwa* (*Hwa*), which means calabash in Chinese, because of the shape of the ventralized embryos. Hwa is a transmembrane protein translated specifically on the dorsal side of the zebrafish blastula with a short extracellular 20 amino acid domain and a larger intracellular domain. Hwa binds Axin1, a key component of the β-catenin destruction complex. It stabilizes β-catenin through the degradation of Axin1 and generates early dorsal signal independently of Wnt growth factors (14, 15). Hwa homologs have been found in echinoderms, *Xenopus,* and reptiles but not in mammals (14).

In this study, we analyzed the sequential signaling steps involved in Spemann–Mangold signaling from egg to tailbud using loss-of-function and gain-of-function approaches in *Xenopus*. *Hwa* mRNA induced complete secondary axes in a process that required β-catenin. Embryos depleted of β-catenin provided a sensitized system to study the subsequent steps. *Xnr* mRNAs induced complete primary axes in β-cat MO-injected embryos. The Nodal extracellular inhibitors *Cerberus-Short* (*CerS*) or *Lefty* rescued trunk and tail axial development in embryos dorsalized by Lithium chloride (LiCl) treatment which consist of radial heads. A single ventral injection of *Chordin* mRNA was able to induce simultaneously D-V and A-P cell differentiation in a dose-dependent way in β-catenin-depleted embryos. Interestingly, this induction took place in the absence of β-catenin/Wnt transcriptional activity measurable by Luciferase reporters. The Wnt antagonist Dkk1 synergized with the early Hwa dorsalizing signal at a later stage, by inhibiting the Wnt signals that normally increase the activity of BMP4 in the ventral center. Depletion of Szl, which increases BMP signaling by increasing Tolloid activity, eliminated the dorsalizing effect of Dkk1. The results suggest a self-organizing pathway for the signaling events that induce dorsal axis development in *Xenopus*.

Results

***Huluwa* mRNA Acts Early in Development.** Microinjection of x*Hwa* mRNA (10 pg) into the marginal zone of a single ventral blastomere at the 4 to 8 cell stage resulted in complete dorsal secondary axes but was unable to rescue any dorsal structures in embryos that had been ventralized by depletion of β-catenin with four injections of β-catenin morpholino (β-cat MO) at 2- to 4- cell stage (Fig. 1 *A*–*H*). This confirmed previous reports of others (14). What was surprising to us was that *Hwa* mRNA reproducibly induced complete axis resulting in two cement glands and four eyes in almost 100% of the cases (*SI Appendix*, Fig. S1 *A*–*D*). This

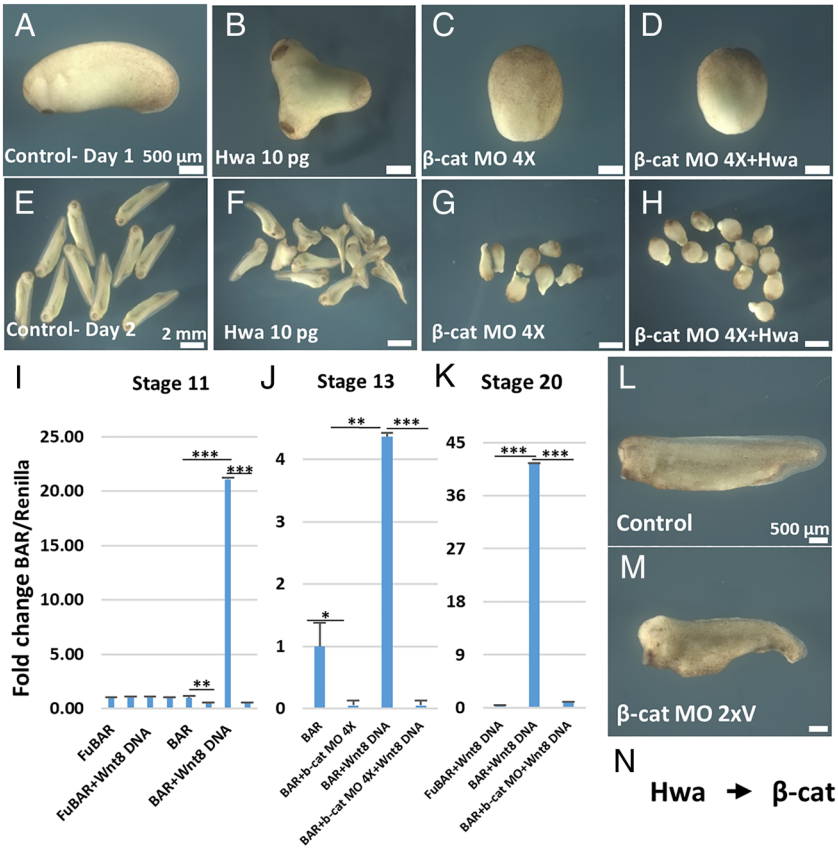

Fig. 1. *Hwa* mRNA did not rescue β-catenin knockdown and β-catenin depletion inhibited Wnt signals through tadpole stages in *Xenopus*. (*A* and *B*) A single ventral injection of *Hwa* mRNA (10 pg) induced complete secondary axes. (*C*) β-catenin depletion with β-cat MO injected four times at 2- to 4-cell caused complete ventralization. (*D*) *Hwa* mRNA 1x ventral failed to rescue the body axis in radially β-cat MO-injected embryos. (*E*–*H*) Group views of the same experiment one day later. (*I*–*K*) Luciferase assays in β-catenin-depleted embryos showing that *xWnt8* DNA was not sufficient to induce BAR transcriptional activity at stage 11, 13, and 20 in β-catenin-depleted embryos. Five embryos were used for each group using biological triplicates. (*L* and *M*) Embryos injected with β-cat MO twice ventral at late four-cell stage inhibited late Wnt signaling with expanded head and cement gland structures. The number of embryos were as follows: (*A*) n = 53, all normal; (*B*) n = 38, all with twinned axes; (*C*) n = 22, all ventralized with a complete absence of dorsal axes; (*D*) n = 18, all ventralized; (*L*) n = 16, all normal; (*M*) n = 20, all with Dkk1-like phenotype with expanded cement gland and enlarged bellies; (*N*) Diagram for Hwa and β-catenin pathway. Error bars indicate SD (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$). (Scale bars: 500 µm for individual embryo photos and 2 mm for group photos.)

high penetrance allowed most of the subsequent work presented here, for it enabled us to inhibit or enhance the secondary axis-inducing activity of reagents coinjected into the same cell as Hwa. Multiple mRNAs active in the Wnt pathway are able to induce twinned axes in *Xenopus*, such as *Wnt1*, *xWnt8*, *β-catenin*, *Dishevelled* (*Dvl*), and dominant-negative *Axin1* (16–20), but none of these have the high penetrance of Hwa in our experience.

Using a β-catenin activated reporter (BAR) (21) luciferase transcriptional assay, we found that *Hwa* mRNA was more active than *Wnt8* mRNA at blastula stages while Wnt8 had comparable activity at gastrula stages (*SI Appendix*, Fig. S1 *E–H*). This difference may be due to the fact that Hwa is a maternal determinant active during early cleavage (14, 22). In *Xenopus*, microinjected DNA is only transcribed after midblastula transition at the 4,000-cell stage (23, 24). It is known that early β-catenin promotes dorsal organizer formation before the onset of gastrulation while after midblastula β-catenin signaling in the ventrolateral domain of the embryo restricts organizer expansion (23). *Hwa* DNA was without phenotypic effect on the embryo while the same amount of *xWnt8* DNA ventralized embryos and *Dkk1* DNA dorsalized embryos causing expanded cement glands and head structures (*SI Appendix*, Fig. S1 *I–L*). The *Dkk1* DNA overexpression phenotype was similar to that of its mRNA (25), suggesting that *Dkk1* mRNA might act at a late stage of development.

xWnt8 DNA strongly induced coinjected BAR (but not the randomized FuBAR control construct) and this was strongly inhibited by β-catenin depletion (Fig. 1*I*). To our surprise, β-cat MO inhibited *xWnt8* DNA activity even at neurula and tailbud stages (Fig. 1 *J* and *K*). This enduring inhibition will become important later when we analyze the effects of microinjected *Xnr* and *Chordin* mRNAs. To confirm that β-cat MO has effects in late development, we injected β-cat MO into 2 ventral blastomeres at the 4-cell stage. We found that the embryos developed with expanded heads, bellies, and cement glands reminiscent of *Dkk1* mRNA injected embryos (Fig. 1 *L* and *M*), suggesting that the β-cat MO inhibits the late xWnt8 signal of the ventrolateral marginal zone (23). This finding is consistent with the original description of the *Xenopus* antisense β-cat MO by Janet Heasman, who found that MO injection into ventral cells at the 8-cell stage induced ectopic cement glands (26).

These results supported the view that the maternal determinant Hwa has a complete requirement for β-catenin (14) (Fig. 1*N*). *Hwa* mRNA acted earlier than *xWnt8* mRNA, and *Hwa* was devoid of phenotypic activity after midblastula transition when injected as DNA.

Nodals Are Sufficient for Complete Axial Induction and Are Required for LiCl Dorsalization. *Xenopus* Nodal-related (Xnr) genes play important roles in mesoderm induction (11, 27). Embryos depleted of β-catenin provide a sensitized system in which axial mesoderm is absent. *Xnr* mRNA injected into the ventral side of wild-type (wt) embryos induced incomplete secondary axes lacking head structures (Fig. 2 *A* and *B*). However, when *Xnr1* (25 pg) was microinjected ventrally into β-catenin-depleted embryos, complete primary axes were induced (Fig. 2 *C–F*). The embryos had trunk-tail structures with somites and heads of various sizes, including greatly enlarged heads and cement glands (Fig. 2*F*). *Xnr6*, a gene expressed at blastula (27), had similar effects, and was able to induce *Chordin* transcripts in in situ hybridization and qRT-PCR analyses of β-catenin-depleted embryos (*SI Appendix*, Fig. S2 *A–D*). The early β-catenin target gene *Siamois* was not affected by *Xnr6*, while the ventral genes *Szl* and *Vent1*, which were elevated in β-catenin-depleted embryos, were inhibited by *Xnr6* (*SI Appendix*, Fig. S2 *E–G*).

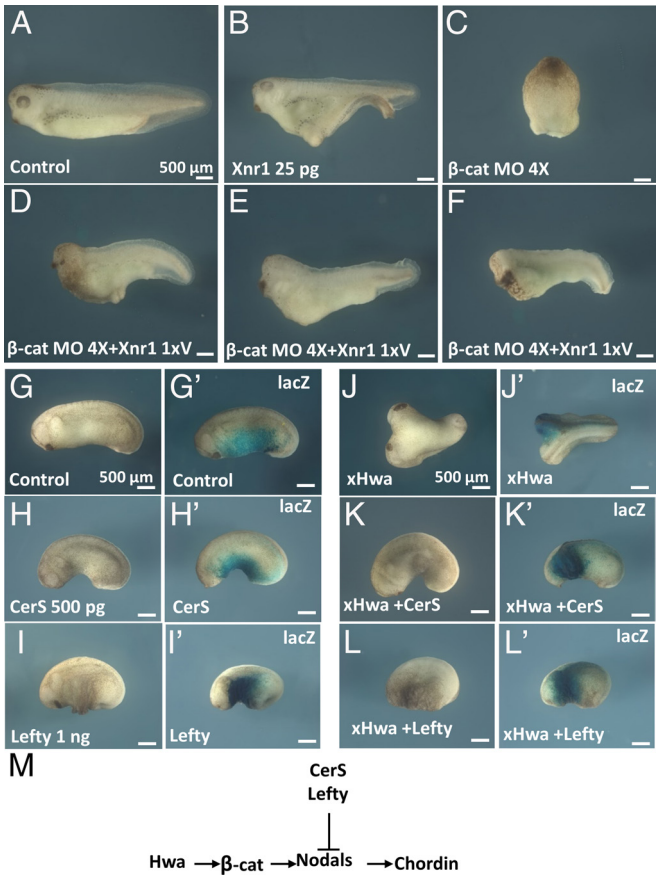

Fig. 2. Microinjection of *Xnr* mRNA into a ventral blastomere rescued a complete dorsal axis in β-catenin-depleted embryos, and the *Xenopus* Nodal-related antagonists *CerS* or *Lefty* mRNA inhibited *Hwa* mRNA ectopic secondary axes in wt embryos. (*A*) Uninjected control embryo. (*B*) *Xnr1* mRNA microinjection induced partial incomplete secondary axes. (*C*) β-cat MO radially injected embryos with complete ventralization. (*D–F*) *Xnr1* (25 pg 1 × ventral) induced complete primary axes in β-catenin-depleted embryos. (*G* and *G'*) Control embryos injected with *LacZ*. (*H–I'*) High doses of *CerS* and *Lefty* caused smaller heads but the primary axis remained. (*J* and *J'*) *Hwa* mRNA injected embryos with twinned axes. (*K–L'*) *CerS* and *Lefty* inhibited secondary axis formation. Results from three independent experiments. (*M*) Diagram of the Hwa/β-catenin/Nodal/Chordin pathway. The number of embryos were as follows: (*A*) n = 48, all normal; (*B*) n = 36, 28 embryos with incomplete axis; (*C*) n = 18, all completely ventralized; (*D–F*) n = 28, 26 embryos with complete axes with large cement gland and 2 ventralized embryos; (*G*) n = 60, all normal; (*H*) n = 20, 14 embryos with no cement gland and small heads and six embryos with smaller heads with cement glands; (*I*) n = 44, all with head ventralization; (*J*) n = 26, all with complete second axes; (*K*) n = 108, 87 with no secondary axes, 17 embryos normal, four embryos with small ectopic heads; (*L*) n = 55, 43 embryos with no axes and 12 embryos with small ectopic bumps. (Scale bars: 500 μm.)

Is Nodal required for axis formation? This is a difficult question to answer in *Xenopus* because there are 5 mesoderm-inducing Nodals and many of them are tandemly repeated in the genome (28). Fortunately, a construct of Cerberus called Cerberus-Short (CerS), consisting of only the cystine knot, is a dedicated inhibitor of Nodals (29). Another organizer gene called *Lefty/Antivin* is an inhibitor of Nodals and also of Activin (30, 31). A single ventral injection of *CerS* (500 pg) or of *Lefty* (1 ng) mRNA allowed primary axis development although head structures were partially inhibited (Fig. 2 *G–I'*). The axis-inducing activity of *Hwa* mRNA could be overcome by coinjection of these high doses *CerS* or *Lefty* mRNA, as seen best in embryos lineage-traced with coinjected *lacZ* mRNA (Fig. 2 *J–L'*).

These results indicate that Xnr growth factors are sufficient for complete axial induction in β-catenin-depleted embryos. The block of Hwa secondary axes by CerS or Lefty indicates that Nodal growth factors are required for formation of the entire dorsal axis in *Xenopus* (Fig. 2*M*).

An influential experiment in *Xenopus* embryology was the treatment of embryos with LiCl at the 32-cell stage (32, 33). LiCl is an inhibitor of GSK3β, a component of the β-catenin destruction complex that stabilizes β-catenin (34). Embryos treated with LiCl developed as dorsalized head structures lacking trunk-tail (Fig. 3 *A* and *B*), which is due to a radial expansion of the Spemann organizer (32). It has been proposed that a gradient of Xnr signals emanating from the endoderm induce both dorsal and ventral mesoderm (11). We now asked whether Nodal signals are required for the formation of the endogenous trunk and tail organizers. A single injection of *CerS* or *Lefty* into the ventral side of embryos treated with LiCl led to development of trunk-tail structures (Fig. 3, compare panel *B* to *D* and *F*). This result indicates that patterning by trunk-tail organizer is mediated by a gradient of Xnr growth factors, and that the radial dorso-anteriorizing effect of LiCl treatment is mediated by increased Xnr signaling (Fig. 3*G*).

Chordin Rescues the Lack of β-Catenin Dose-Dependently. We next tested whether Chordin, a dedicated BMP antagonist induced by high levels of Nodal signal from the organizer (6) could rescue axial structures in β-catenin-depleted embryos (Fig. 4 *A* and *B*). At low doses of *Chordin* mRNA (1 or 2 pg), only trunk-tail structures were induced (Fig. 4*C*). At higher concentrations of *Chordin* mRNA (5, 10, 25, or 50 pg), axes with complete D-V and A-P structures, including eyes, cement glands, and somites, and trunk-tail structures were induced at high frequency (Fig. 4 *D–F*). At high levels of *Chordin*, head structures were expanded with respect to wt (Fig. 4, compare *A* to *F*). We note that the head regions rescued in β-catenin-depleted embryos by *Chordin* (and also by *Nodal*) were more pigmented than in wt (Fig. 4 *D* and *E*; also Fig. 2 *D* and *F*). This was because the entire axis was respecified by 1 × ventral injection with embryos developing the organizer on the more pigmented injected side. In molecular analyses, CNS tissue was induced in this sensitized system, as indicated by the panneural marker *Sox2* (*SI Appendix*, Fig. S3 *A–C*). The forebrain and hindbrain marker *Otx2* was also induced (*SI Appendix*, Fig. S3 *D–G*). By qRT-PCR at the early neurula stage, the eye marker *Rx2a* and the panneural marker *NCAM* were strongly induced with respect to β-catenin-depleted embryos (*SI Appendix*, Fig. S3 *H* and *I*). Noggin, another important anti-BMP secreted by the organizer (35), was also able to rescue the β-catenin depletion phenotype (*SI Appendix*, Fig. S3 *J–L*).

The rescue of entire dorsal axes by Chordin or Xnr had one remarkable aspect shown in Fig. 1 *I–K*. The depletion of β-catenin by β-cat MO was enduring, inhibiting xWnt8-induced BAR-Luciferase activity at mid-gastrula, early neurula, and tailbud (36) despite having been injected much earlier at 2- or 4-cell stage. Since canonical Wnt signaling has a complete requirement for β-catenin function, these results indicate that, surprisingly, complete embryonic axes including head, trunk, and tails can be obtained in the absence of canonical Wnt signaling.

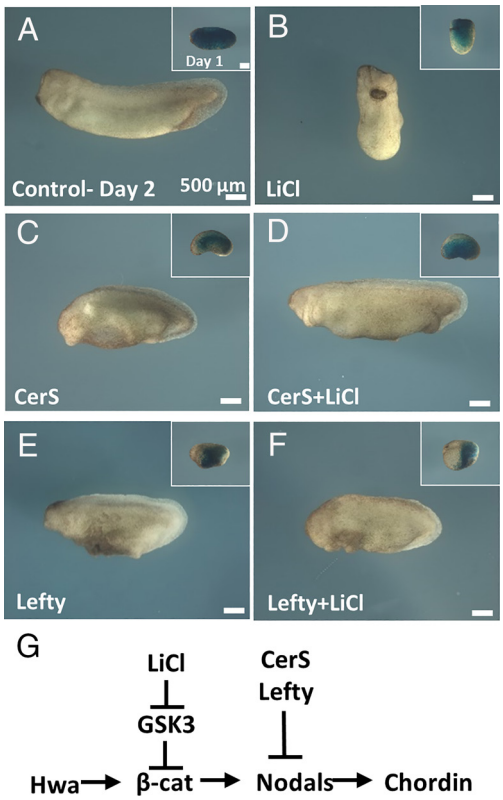

Fig. 3. The trunk-tail organizer was rescued by a single injection of the Nodal antagonists *CerS* or *Lefty* in LiCl dorsalized embryos consisting of head structures. (*A*) Control embryos with 1× ventral *lacZ* mRNA staining in *Inset*. (*B*) Embryos treated with LiCl at the 32-cell stage have radial heads lacking trunk-tails. (*C*) *CerS* single ventral injection caused ventralization with small heads. (*D*) CerS restored trunk and tail structures in LiCl-treated embryos. (*E*) Ventralized embryo injected with *Lefty* mRNA. (*F*) *Lefty* mRNA single ventral injection at the four-cell stage rescued the LiCl effect increasing trunk-tail structures. Results from three independent experiments. (*G*) Diagram for the Hwa/β-catenin/Nodal/Chordin pathway. The number of embryos were as follows: (*A*) n = 163, all normal; (*B*) n = 151, all with strong dorsalization; (*C*) n = 137, all ventralized; (*D*) n = 123, 71 embryos with trunks and small heads, 52 embryos were partially dorsalized but less than LiCl; (*E*) n = 68, all with small heads; (*F*) n = 67, all with trunk-tails and small heads. (Scale bar, 500 µm.)

The Hwa Pathway Leads to BMP Inhibition and Synergizes with Dkk1. The pathway downstream of Hwa axial induction is compatible with a pathway of consecutive signaling events shown in Fig. 4*G*. We therefore tested whether overexpressed mBMP4 could counteract the effects of *Hwa* mRNA in coinjection experiments. Moderate amounts of *mBMP4* mRNA (50 pg), which ventralized the primary axes only partially, were able to eliminate the secondary Hwa axis when coinjected into the same cell (*SI Appendix*, Fig. S4 *A–I*). The results suggest that *Hwa* mRNA inhibits BMP signaling through a series of sequential signaling events.

The Spemann organizer secretes not only BMP and Nodal inhibitors but also many Wnt inhibitors, of which Dkk1 is the most famous (25, 37, 38). In coinjection experiments, we found a robust synergy between Hwa and Dkk1 (Fig. 5 *A–D*). A single ventral injection of Dkk1 (50 pg) produced embryos with enlarged heads, bellies, and cement glands. When Dkk1 was coinjected together with *Hwa* mRNA, trunk structures were greatly reduced and head structures expanded, producing embryos consisting of two large heads with a single, almost radial, cement gland (Fig. 5*D*). In lineage tracing experiments, ventrally injected Dkk1 cells remained in the trunk ventral region, while when coinjected with Hwa cells were fated to the superficial layers of the expanded head structures (*SI Appendix*, Fig. S5 *A–D*). By in situ hybridization of *Sox2*, Dkk1 increased CNS and in combination with Hwa induced radially expanded brains (Fig. 5 *A'–D'*). At mid gastrula, Hwa and Dkk1 increased *Chordin* expression, forming two expanded organizers that did not become radial (*SI Appendix*, Fig. S5 *E–H*). Another Wnt inhibitor secreted by the organizer is Frzb-1 which when overexpressed causes enlarged heads and cement glands like Dkk1 (39). Frzb-1 also cooperated with Hwa in coinjection experiments but had weaker phenotypes than Dkk1 that never reached radial cement glands (*SI Appendix*, Fig. S5 *I–L*). The results are consistent with previous work showing that the early Hwa signal does not involve extracellular Wnt growth factors, which instead have a ventralizing effect later in development (14, 23).

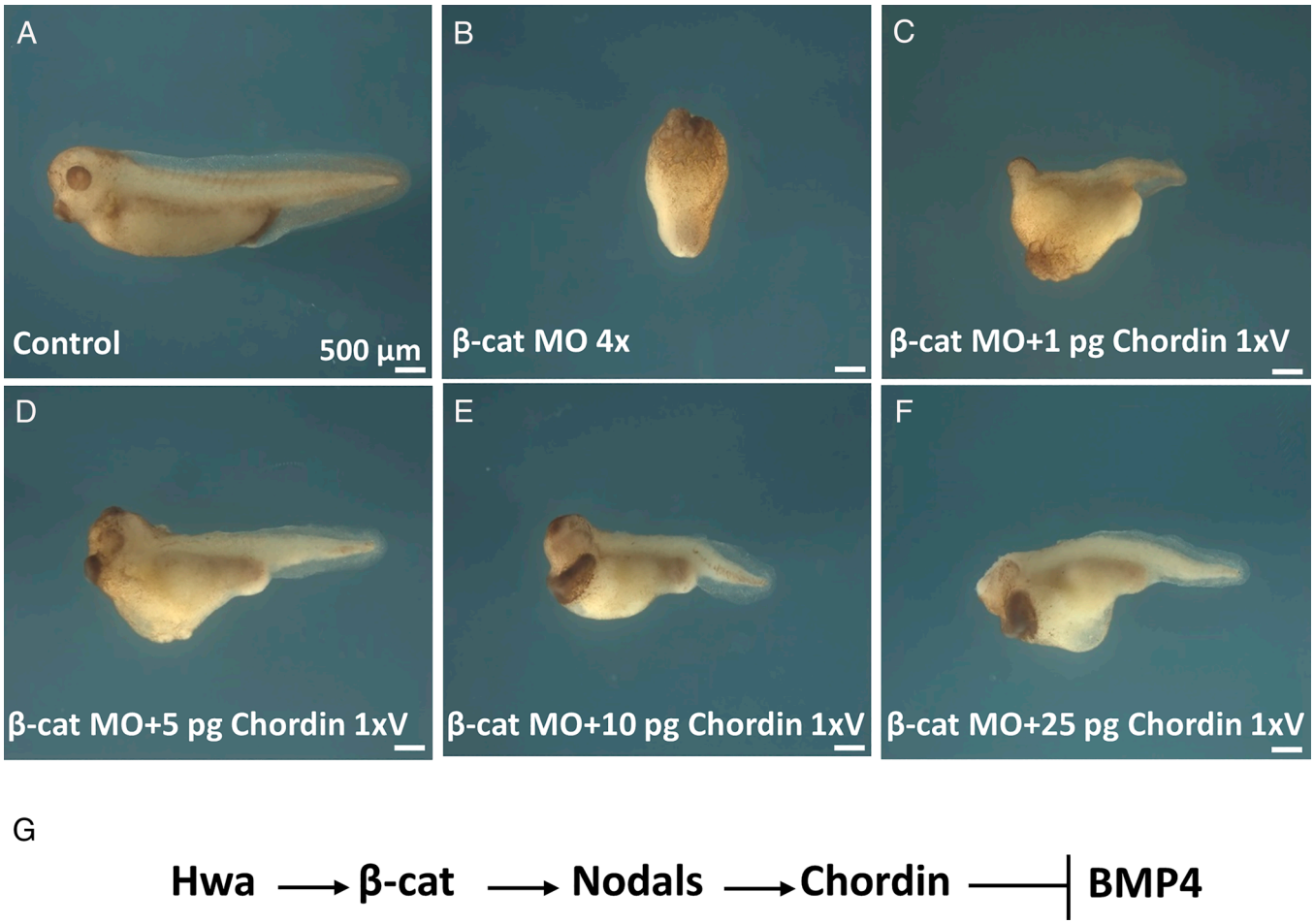

Fig. 4. *Chordin* mRNA rescued both D-V and A-P axial structures in a dose-dependent manner. (*A*) Control embryo. (*B*) Embryos with 4 x injection of β-cat MO (34 pg total) were completely ventralized. (*C–F*) *Chordin* mRNA injection rescued A-P and D-V axial structures in β-catenin-depleted embryos in a dose-dependent way; note that phenotypes in both axes are intertwined. Results from three independent experiments. (*G*) Diagram of the Hwa/β-catenin/Nodal/Chordin/BMP4 pathway. The number of embryos were as follows: (*A*) n = 176, all normal; (*B*) n = 131, all ventralized; (*C*) n = 18, 1 embryo with weak axis and others ventralized; (*D*) n = 38, all with complete axes including eyes and cement glands; (*E*) n = 21, with expanded cement gland; (*F*) n = 118, all with very large CNS, eyes, and cement glands. In addition, experiments were performed for β-cat MO 4 x + 2 pg of Chordin, n = 16, 7 embryos with incomplete axes with no cement gland, 4 with cement gland and 5 with no axis; and β-cat MO 4 x + 50 pg of Chordin 1 x ventral, n = 14, all with strong axial rescue. (Scale bars: 500 µm.)

The Tolloid Inhibitor Sizzled Is Required for the Synergy between Hwa and Dkk1. The strong dorsalization observed in Hwa plus Dkk1 single ventral injections suggested to us that inhibition of the BMP pathway might be involved. Szl is a competitive inhibitor of the metalloproteinase Tolloid produced in the ventral center of the *Xenopus* gastrula (7). *Xenopus* Xolloid-related (Xlr) enzymes degrade Chordin on the ventral side of the embryo, resulting in increased BMP signaling (7, 37). An excellent antisense MO for Szl exists (40), which when injected into each blastomere of the 4-cell embryo leads to ventralization with small heads and expanded ventro-posterior tissues (Fig. 5 *E–F'*). In Szl-depleted embryos, a single injection of Hwa plus Dkk1 could not overcome the ventralized Szl MO phenotype (Fig. 5 *E–H*). Szl MO was also able to reverse the radial CNS induction caused by the synergy between Hwa and Dkk1 (Fig. 5 *G'* and *H'*). These findings indicate that Szl, a component of the Chordin/Tolloid/BMP pathway, is required for the synergistic effects of Hwa plus Dkk1.

Szl MO was able to eliminate the phenotypic dorsalizing effects of *Dkk1* mRNA overexpression (*SI Appendix*, Fig. S6 *A–D*). This suggested that the big head and cement gland phenotype of Wnt inhibition is due to decreased of BMP signaling in the wt embryo. To test this, we used coinjection experiments of *xBMP4* and *Dkk1* mRNAs together with *BMP response element-Luciferase (BRE-Luc)* DNA (41) and *Renilla* mRNA (42) into a single ventral blastomere of wt embryos. xBMP4 increased reporter activity and the Wnt inhibitor Dkk1 was unable to affect this at early gastrula stage 10 (*SI Appendix*, Fig. S6*E*, compare lane 3 to 4). However, at the early neurula stage, Dkk1 was able to inhibit both endogenous BMP and xBMP4-induced signaling (*SI Appendix*, Fig. S6*F*, compare lane 1 to 2, and 3 to 4). We conclude that the organizer Wnt inhibitor Dkk1 caused BMP inhibition at later stages of development but not at early gastrula. Fig. 6 summarizes a possible signaling pathway for vertebrate axial development derived from the experiments presented in this study.

Discussion

Huluwa Signals Early in Development. Hwa is a maternally encoded transmembrane protein expressed in the dorsal side of zebrafish and *Xenopus* embryos that stabilizes β-catenin independently of Wnt growth factor signals (14). We confirmed the complete requirement of β-catenin for the potent axis inducing activity of *Hwa* mRNA. *Wnt* mRNAs can also induce complete secondary axes (16, 45) but we showed here that *Hwa* mRNA induced β-catenin transcriptional activity earlier than *xWnt8* mRNA. When microinjected as DNA, which is expressed after midblastula transition, *Hwa* was devoid of activity, in contrast to *xWnt8* or *Dkk1* DNA. These early effects of Hwa are consistent with its proposed role as the true dorsal maternal signal (14).

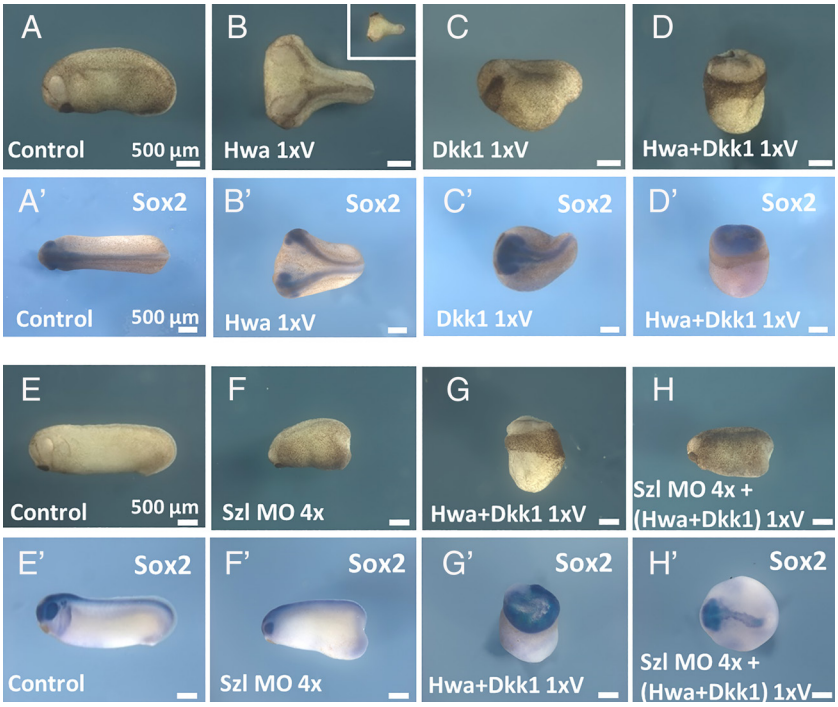

Fig. 5. The Wnt antagonist Dkk1 synergized with *Hwa* mRNA in dorso-anterior CNS development, and Szl depletion blocked this effect. (*A*–*D*′) *Hwa* mRNA in combination with *Dkk1* mRNA had striking anteriorized CNS structures with almost radial cement gland and lacking trunk-tail structures. (*E* and *E*′) Uninjected control embryo. (*F* and *F*′) 4 x Szl MO injection caused strong ventralization with small heads and expanded ventro-posterior tissues. (*G* and *G*′) Embryo coinjected with *Hwa* and *Dkk1* with radial cement bland and anterior CNS. (*H* and *H*′) Szl depletion strongly antagonized the synergy between *Hwa* and *Dkk1*. Results from three independent experiments. Numbers of embryos analyzed were as follows: (*A* and *A*′) n = 59, all normal; (*B* and *B*′) n = 46, 45 with twinned axes; (*C* and *C*′) n = 46, 45 with strong dorsalization; (*D* and *D*′) n = 77, all strongly dorsalized; (*E* and *E*′) n = 131, all normal; (*F* and *F*′) n = 43, all ventralized with small heads and expanded ventral tissues; (*G* and *G*′) n = 65, 33 embryos with radial cement glands and no trunk, 32 embryos with shortened double axes with cement glands covering over 50% of the radius of the embryo; (*H* and *H*′) n = 41, 38 embryos were ventralized with the Szl MO phenotype of small heads and cement glands, two embryos with residual large cement glands, one embryo with two weak axes. (Scale bars: 500 µm.)

In a Sensitized β-Catenin Depleted System, *Xenopus* Nodal-Related Genes Rescue Complete Embryonic Axes. Dorsal β-catenin signaling has been proposed to induce the Nieuwkoop signaling center in endoderm, which by secreting Xnrs in turn induces the Spemann organizer at early gastrula (11, 29). Nodals/TGF-βs signal through Smad2/3 transcription factors that activate the expression of organizer genes such as Chordin (42, 46). *Xnr* mRNAs were able to induce only partial secondary axes when injected into a ventral blastomere at the 8-cell stage. However, the response is much greater in β-cat MO embryos. Complete axial structures were induced including head and trunk-tail with some embryos with enlarged heads and cement glands (Fig. 2 *A*–*F*). Xnrs were also shown to be required for axial formation, as revealed by coinjection experiments in which the Nodal inhibitors CerS or Lefty (29–31) blocked development of the entire Hwa secondary axis. Treatment of *Xenopus* embryos at the 32-cell stage with LiCl results in radial organizers with embryos consisting of heads without trunk-tail structures (32). We found that a single ventral injection of Xnr inhibitors restored the development of trunk-tails in LiCl-treated embryos. This supports the proposal that a gradient of Xnrs induces both head and trunk-tail development (11, 12).

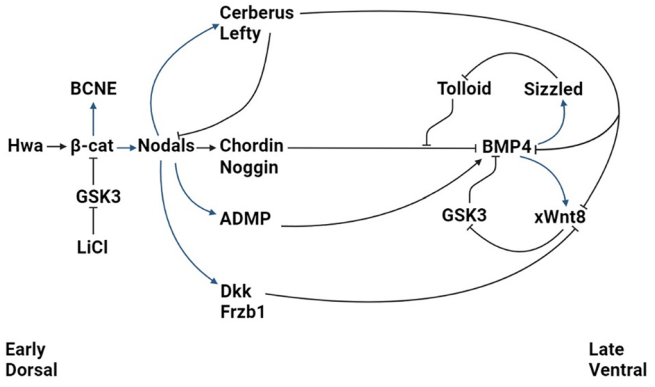

Fig. 6. Proposed molecular pathway for self-organizing axial development in *Xenopus* embryos. Black activator or inhibitory arrows indicate interactions at the protein level and blue arrows show that transcriptional activation steps. Hwa is an egg maternal mRNA that stabilizes the β-catenin transcriptional activator by increasing Axin1 degradation (14). Dorsal β-catenin promotes high Nodal signaling levels from the Nieuwkoop center in endoderm at early blastula, as well as the BCNE (Blastula Chordin and Noggin Expression center) in dorsal ectoderm (43). The BCNE expresses Siamois, a homeobox protein that plays an important role in the induction of Spemann organizer genes but is not indicated in this diagram (44). Expression of *Xenopus* Nodal-related genes is required for the induction of secondary axes by Hwa. At the gastrula stage, the Spemann–Mangold organizer center secretes a cocktail of proteins such as the BMP antagonists Noggin and Chordin, the Wnt antagonists Dkk1 and Frzb-1, ADMP, and the multivalent inhibitors Lefty (antagonist of Nodals and activing) and Cerberus (antagonist of Nodal, BMP, and Wnt). At the opposite pole of the embryo, the ventral center has high BMP4/7 signals that oppose the influence of the organizer. xWnt8 is induced by BMP and, in a positive feedback loop, increases BMP signaling through GSK3 inhibition. Sizzled is an inhibitor of the Tolloid proteinase that degrades Chordin, which is transcribed at high levels of BMP, forming a negative feedback loop that increases Chordin levels when BMP is high. Dkk1 and Frzb-1 inhibit the activity of xWnt8 at late gastrula, reducing BMP signaling.

High Levels of β-Catenin Signaling Are Not Required for the Induction of Complete Axial Structures by Chordin. The next step after Nieuwkoop center signaling is the formation of the Spemann–Mangold organizer in mesoderm at early gastrula. This tissue secretes a cocktail of growth factor inhibitors including anti-BMPs such as Chordin and Noggin, anti-Wnts such as Dkk1 and Frzb-1, and multivalent inhibitors of Nodals and other growth factors such as Cerberus and Lefty (Fig. 6) (5). *Chordin* mRNA in wt embryos induces principally incomplete axes lacking head structures in single ventral injections. However, Chordin had a remarkable dose-dependent effect when microinjected into β-catenin-depleted embryos. Trunk-tail structures with somites were generated at low levels, and at higher concentrations, complete heads with large eyes and expanded cement glands were also induced (Fig. 4).

At first, we thought that the effects of β-cat MO might wear off as development progressed. However, transcriptional reporter gene assays showed that β-cat MO blocked xWnt8 signaling as measured through the BAR Luciferase reporter assay (21) during gastrula, neurula, and tailbud (Fig. 1 *I*–*K*). This is very puzzling since Wnt/β-catenin is one of the main pathways regulating vertebrate development (47). The implication would be that β-catenin

is required only for the initial Hwa signal, and dispensable for the subsequent induction of the main body regions. However, it should be kept in mind that morpholino knockdown may only partially decrease β-catenin levels. In the embryo, *β-catenin* mRNA levels are very high and not affected by depletion by MO (48) and it is therefore likely that some β-catenin protein will persist. Recent work from the Hoppler lab has shown that zygotic β-catenin protein binds to 10,000 chromatin sites in the *Xenopus tropicalis* genome while fewer than 200 genes are activated by xWnt8 and depend on additional signals such as those provided by BMP or FGF signaling (49, 50). Thus, while β-cat MO prevents detectable BAR Luciferase signaling in our experiments, low levels of β-catenin might still be bound to chromatin throughout the genome. Why do Chordin and Nodals induce heads only in the sensitized β-catenin-depleted embryos? One possible explanation, which remains to be tested, is that the organizer also secretes ADMP (51), as well as BMP2 (52), which inhibits head structures. An intriguing feature of the rescue of β-catenin-depleted embryos by a single injection of anti-BMP was that the A-P and D-V axes were always intertwined; the mechanistic node of connection between these two patterning systems is deserving of future study.

Huluwa and Dkk1 Cooperate. The organizer secretes Wnt antagonists such as Dkk1 and Frzb-1 that generate dorsalized phenotypes with large heads, bellies, and cement glands, but do not induce second axes (25, 39). It had been previously shown that the *Hwa* mRNA effect is not inhibited by Dkk1 in *Xenopus*, demonstrating that the maternal signal is independent of secreted Wnt growth factors (14). Using higher amounts of *Dkk1*, we now found a strong synergy with *Hwa* mRNA leading to embryos with almost circular cement glands and CNS in many cases, and two double heads lacking trunk-tails in others. *Frzb-1* mRNA also increased the Hwa phenotype but was weaker than Dkk1. In zebrafish, as detailed in the supplemental information of Yan et al., coinjection of Hwa and Wnt antagonists led to increased Chordin expression and dorsalization (14), and the present results agree with this.

The Tolloid Inhibitor Sizzled Is Required for Dorsalization by Dkk1. Hwa-induced secondary axes were suppressed by coinjection of BMP4. The phenotype of the combination of Hwa and Dkk1 mRNAs, and to some extent that of Dkk1 alone, resemble embryos with low BMP signaling. We found that in BMP luciferase reporter gene assays Dkk1 inhibited BMP at late gastrula/early neurula but not at early gastrula. In the embryo, the D-V patterning system depends on the BMP4/Chordin/Tolloid/Sizzled biochemical pathway (Fig. 6). Szl is a secreted Frizzled-related protein (sFRP) that has lost Wnt regulating activity (40, 53) and serves as a competitive inhibitor of Tolloids, which are metalloproteinases that cleave and inactivate Chordin leading to increased BMP signaling on the ventral side (7, 8). In zebrafish, the Ogon/Szl mutation has been studied extensively and found to act at a relatively late stage of D-V patterning (54, 55). In *Xenopus*, we found that Szl was required for the synergy between Hwa and Dkk1. In addition, the dorsalizing effects of Dkk1 alone were also overcome by Szl MO. In genetic terms, Szl is epistatic over Dkk1 or Hwa/Dkk1 overexpression. The cooperation between Hwa and Dkk1 is likely mediated by the expansion of the Chordin domain at late gastrula. This is because when Szl is depleted, Tolloid increases, and Chordin is degraded, leading to an increase in BMP in the embryo. These epistatic experiments indicate that Szl acts downstream or in parallel to Dkk1 (Fig. 6).

The phenotypic effects of Dkk1 and Frzb-1 overexpression could be mediated by a variety of mechanisms. For example, an xWnt8 target gene could inhibit and limit the Chordin expression domain. An interesting possibility would be through the inhibition of phosphorylations by GSK3 on Smad1/5/8. It is known that the termination of the activity of BMP receptor transcription factors Smad1/5/8 is mediated by GSK3 phosphorylations (56, 57). Dkk1 could increase Smad1/5/8 GSK3 phosphorylations at late gastrula by inhibiting xWnt8, leading to a decrease in the duration of BMP signals (Fig. 6). We have hypothesized earlier, but never demonstrated, that the Chordin/Szl/Tolloid/BMP pathway could be involved in regulating the intensity BMP signaling in the D-V axis, and that Dkk1/Wnt8/GSK3 might regulate the duration of the Smad1/5/8 signal in the A-P direction (58). The analysis of this mechanism would be complicated because the duration of the Smad4 signal, which is shared by both the Nodal/TGF-β/Smad2/3 and the BMP/Smad1/5/8 signals, is also regulated by GSK3 phosphorylations (42, 59). Another area that would deserve further exploration is the molecular mechanism by which Smad2/3 and Smad1/5/8 oppose each other in the embryo (60, 61). Related studies in zebrafish have shown that the opposition between Nodal and BMP signals is crucial to the formation of the D-V, A-P, and left–right body axes (62–64). Signal integration is a hallmark of organizers, which pattern embryonic structures as an integrated whole, and further studies in the zebrafish and *Xenopus* systems will be needed to identify the key nodes of interaction between the three embryonic axes.

In sum, the availability of Hwa as the dorsal maternal determinant provided a robust experimental tool that allowed molecular dissection of the axis formation pathway through the coinjection experiments described in this paper. Several unexpected conclusions emerged. For example, both head and tail organizers are induced by graded Nodal signals, and the dorsalizing effect of LiCl is mediated by Nodal signaling. In β-cat MO embryos, Nodal or its target gene Chordin induced complete axes in which D-V and A-P patterning are intimately intertwined. In addition, Wnt inhibitors secreted by the Spemann–Mangold organizer function late in the axial development pathway by regulating the BMP/Chordin/Tolloid/Sizzled signaling pathway (Fig. 6).

Materials and Methods

***Xenopus* Ethic Statements.** Animal experiments were performed according to the recommendations in the Guide for the "Care and Use of Laboratory Animals of the NIH." All institutional Animal Care and Use Committee (IACUC) protocols (#D16-00124) of the University of California Animal Research Committee Los Angeles were followed (Medical School - Permit Number: ARC-1995- 129). Mature pigmented females and male albino *X. laevis* were purchased from Xenopus-1 Inc.

***Xenopus* Embryo Manipulations and LiCl Treatment.** Unfertilized eggs were collected in cages containing 2 liters of 1 × high salt solution (diluted from a 10× stock containing 70 g of NaCl, 1.8 g of KCl, 3.5 g of $CaCl_2(2H_2O)$ and 2.4 g $MgCl_2$, HEPES 4.3 g per liter (pH adjusted to 7.6 with NaOH). Eggs were collected with a 25 mL pipette and placed in 100 × 20 mm plastic dishes (Fisher FB0875711Z) with a minimal amount of liquid. Eggs were then fertilized in vitro with freshly dissected testes. One testis was mashed up with a loose pestle in 800 μL of 1 × MMR (Marc's modified Ringers; 100 mM NaCl, 2 mM of KCl, 2 mM $CaCl_2(2H_2O)$, 1 mM $MgCl_2$, and 5 mM HEPES (pH adjusted to 7.4 with NaOH). 200 μL of sperm solution was distributed on the eggs with a Gilson pipette and then gently mixed with the same pestle used to homogenize the testis, sometimes supplemented by brushing a fragment of testis over the eggs, and allowed to rest for 5 min at room temperature (RT). Then, 0.1 × MMR was added to the Petri dishes and not moved for 70 min at RT or for 2 h at 15 °C. Fertilized embryos were then dejellied with freshly made 2% Cysteine solution in 0.1 x MMR (adjusted to pH 7.8 with NaOH) for 10-15 min at RT. Next, embryos were washed 3 times with 0.1 x MMR. Embryos were injected in 1 x MMR in 60 mm x 15 mm dishes (Fisher

Table 1. Primer sequences for qRT-PCR

Gene	Forward primer	Reverse primer
ODC	CAGCTAGCTGTGGTGTGG	CAACATGGAAACTCACACC
Chordin	CCTCCAATCCAAGACTCCAGCAG	GGAGGAGGAGGAGCTTTGGGACAAG
Siamois	AAGATAACTGGCATTCCTGAGC	GGTAGGGCTGTGTATTTGAAGG
Sizzled	GTCTTCCTGCTCCTCTGC	AACAGGGAGCACAGGAAG
Vent1	GGCACCTGAACGGAAGAA	GATTTTGGAACCAGGTTTTGAC
Otx2	GGATGGATTTGTTACATCCGTC	CACTCTCCGAGCTCACTTCCC
Rx2a	AGACTGGTGGCTATGGAG	ATACCTGCACCCTGACTT
NCAM	GCGGGTACCTTCTAATAGTCAC	GGCTTGGCTGTGGTTCTGAAGG

FB0875713A) coated with 2% agar (Fisher, BP1424-500) in H_2O and transferred to 0.1 × MMR agar dishes. After completion of the experiment embryos were incubated at 15 °C overnight (taking care embryos were uniformly distributed in the Petri dish), which slows down development and improved survival. Embryos were staged using the Nieuwkoop normal table (36) and fixed in 0.5 x MEMFA (0.05 M MOPS pH 7.4, 1 mM EGTA, 0.5 mM $MgSO_4$, and 3.7 % formaldehyde) in 1 Dram vials (Fisher 03-339-25B). Embryos were treated with 300 mM of LiCl in 0.1 x MMR for 7 min at the 32-cell stage in dishes without agarose. Embryos were transferred three times into fresh 0.1 x MMR agar dishes and cultured at 15 °C overnight.

DNA Injections and Morpholinos. pCS107-Hwa, pCS2-Wnt8, pCS2-Dkk1, pGL3-BRE-Luc, pGL3-BAR-Luc, and pGL3-FuBAR-Luc constructs were microinjected as 20 pg in 4 nl into a single ventral blastomere or each blastomere at the 4-cell stage, as indicated. *X. laevis* antisense β-cat MO (5′TTTCAACCGTTTCCAAAGAACCAGG 3′) and Szl MO (5′GAGGAGCAGGAAGACTCCGGTCATG 3′) were purchased from Gene Tools, LLC (Philomath, OR, USA). 8.5 ng of β-cat MO was injected 4 times radially (total of 34 ng) at the 4-cell stage, or 2 times into ventral blastomeres (total 17 ng). 8.5 ng of Szl MO was injected into each blastomere (total 34 ng) at the 4-cell stage.

mRNA Synthesis and Injection. pCS2-*Chordin*, pCS2-*Dkk1*, pCS2-*Frzb-1*, pCS2-*Xnr1*, pCS2-*Xnr6*, pCS2-*Cerberus-short*, pCS2-*Antivin/Lefty*, pCS2-*nLacZ*, and pCS2-*Renilla* were linearized using NotI. pCS107-*xHwa* (a gift of Qinghua Tao) was linearized with Hpa1, pSP35-*Noggin* with EcoRI, pSP64T-*xBMP4* with SacI, and pCS2-*mBMP4* with Asp718. mRNAs were synthesized using SP6 RNA polymerase and the mMessage mMachine SP6 kit (Invitrogen). They were purified with the MegaClear kit (Invitrogen). Synthetic mRNAs were injected into a single ventral blastomere at the 4- or 8-cell stage at the indicated amounts in a volume of 4 nL.

Luciferase Assay in Embryo. Experiments were performed with five embryos per sample in triplicate. BRE-Luc DNA, BAR-Luc DNA, or FuBAR-Luc DNA plus *Renilla* mRNA (42). Groups of injected embryos were lysed at stages 8, 9, 10, 11, 13, and/or 20 by pipetting up and down in 150 μL of 1 × lysis buffer (Promega) for 20 min at RT. Samples were then centrifuged at 2,000 rpm for 5 min at RT and 20 μL of supernatant was used from each assay. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using the Glomax Luminometer (Promega). Luciferase values of each sample were normalized for Renilla activity.

LacZ Staining and In Situ Hybridization. Embryos were fixed with 0.5 x MEMFA for 1 h and washed three times with PBS (Fisher Scientific, BP3994) for 10 min each. Embryos were then stained with X-Gal solution [1 mM X-Gal, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, in PBS] overnight at 4 °C. Then, they were washed two times with PBS for 5 min. After refixation in 0.5 x MEMFA at room temperature for 2 h, embryos were washed three times with PBS, for 5 min. In situ hybridizations were performed as described previously at https://www.hhmi.ucla.edu/derobertis. Embryos were imaged using a ZEISS Axio Zoom. V16 dissecting microscope using the Z-stack function.

qRT-PCR. The qRT-PCR analyses were performed using *Xenopus* embryos at stages 11.5 or 13 as previously described (65). Primer sequences for qRT-PCR are listed in Table 1.

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

Data, Materials, and Software Availability. All study data and materials are included in the article and/or *SI Appendix* and available upon request. No novel software or reagents were generated in this study.

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