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Chordin Is Required for the Spemann Organizer Transplantation Phenomenon in *Xenopus* Embryos

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Summary

We analyzed the Chordin requirement in *Xenopus* development. Targeting of both *chordin Xenopus laevis* pseudoalleles with morpholino antisense oligomers (Chd-MO) markedly decreased Chordin production. Embryos developed with moderately reduced dorsoanterior structures and expanded ventroposterior tissues, phenocopying the zebrafish *chordino* mutant. A strong requirement for Chordin in dorsal development was revealed by experimental manipulations. First, dorsalization by lithium chloride treatment was completely blocked by Chd-MO. Second, Chd-MO inhibited elongation and muscle differentiation in Activin-treated animal caps. Third, Chd-MO completely blocked the induction of the central nervous system (CNS), somites, and notochord by organizer tissue transplanted to the ventral side of host embryos. Unexpectedly, transplantations into the dorsal side revealed a cell-autonomous requirement of Chordin for neural plate differentiation.

Introduction

The dorsal lip, or Spemann organizer, of the amphibian gastrula has remarkable inductive properties. When grafted into the ventral side of a host embryo, this small group of cells can induce a second embryonic axis—including a central nervous system (CNS), dorsal mesoderm, and secondary gut—in neighboring host cells. Considering these striking properties, isolating the molecules that mediate these cell-cell inductions has been an intensive area of research (reviewed in De Robertis and Aréchaga, 2001). A large number of candidate secreted proteins specifically expressed in the organizer region have been isolated (Harland and Gerhart, 1997; De Robertis et al., 2000). Surprisingly, many of these factors encode secreted antagonists of growth factor signaling. These include bone morphogenetic protein (BMP) inhibitors, such as Chordin, Noggin, Follistatin, and Xnr3; Wnt inhibitors, such as Dkk, Frzb-1, and Crescent; Nodal signaling inhibitors, such as Lefty/Antivin; and inhibitors of multiple signaling pathways, such as Cerberus, an inhibitor of BMP, Wnt, and Nodal signals. In overexpression studies in *Xenopus*, all of these molecules have potent effects on the differentiation of embryonic cells. In order to unravel the respective contributions of each antagonist to the organizer phenomenon, one should ablate the function of each individual gene in the grafted tissue. Here we test the requirement for Chordin in *Xenopus* development.

Chordin is a large secreted protein expressed specifically in the organizer, which, when overexpressed, can recapitulate most of the activities of the organizer (Sasai et al., 1994, 1995). Chordin protein is expressed abundantly, reaching extracellular concentrations of 6–12 nM in the dorsal lip (Piccolo et al., 1996). Chordin directly binds BMPs via cysteine-rich modules (called CRs), preventing BMPs from binding to their cognate receptors (De Robertis et al., 2000). In *Drosophila*, the Chordin homolog Short gastrulation (Sog) is expressed in ventral neuroectoderm and is required for CNS development and dorsal-ventral patterning of the embryo (François et al., 1994; Srinivasan et al., 2002). The conservation extends to cofactors of Chordin/Sog, in particular, the metalloproteinase Xolloid/Tolloid, which cleaves Chordin/Sog, and the BMP binding protein Twisted gastrulation (Tsg), which forms ternary complexes with Chordin/Sog and BMPs (Piccolo et al., 1997; Marqués et al., 1997; Oelgeschläger et al., 2000; Chang et al., 2001; Ross et al., 2001).

In zebrafish, the Chordin homolog *chordino* was identified as the strongest ventralized mutant resulting from saturation genetic screens (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). In *chordino* mutants dorsal marker gene expression is reduced and ventral markers are expanded in the mesoderm and ectoderm at the gastrula stage. Although *chordino* embryos recover at later stages, they develop with small heads and eyes, U-shaped somites, shortened trunk, and increased ventroposterior tissues, including blood islands, before dying after three days of development. These zebrafish studies demonstrated that Chordin is required for dorsal-ventral patterning during gastrulation. In the mouse, the situation is different, for most $chordin^{-/-}$ embryos have a normal CNS. Mutant embryos lack anterior notochord, and the pharyngeal endoderm, which expresses Chordin, is greatly reduced. The pharyngeal defect results in a set of head, neck, and cardiac malformations that mimic human DiGeorge syndrome. In a small percentage of embryos, a ventralized phenotype at gastrula was seen, in which the ventral mesoderm giving rise to the allantois was expanded at the expense of intraembryonic mesoderm (D. Bachiller, J. Klingensmith, and E.D.R., submitted). Although the mouse *chordin* mutant behaves differently from the zebrafish with respect to CNS development, when the BMP antagonist *noggin* is also mutated, the forebrain does not develop, indicating that Noggin can partially compensate for the lack of Chordin (Bachiller et al., 2000).

In the present study we downregulated the expression of Chordin protein in *Xenopus* using morpholino antisense oligomers (Summerton, 1999; Heasman, 2002). The overall phenotype resembled that of the *chordino* mutation, with reduction of dorsoanterior, and expansion of ventroposterior, structures in the embryo. The

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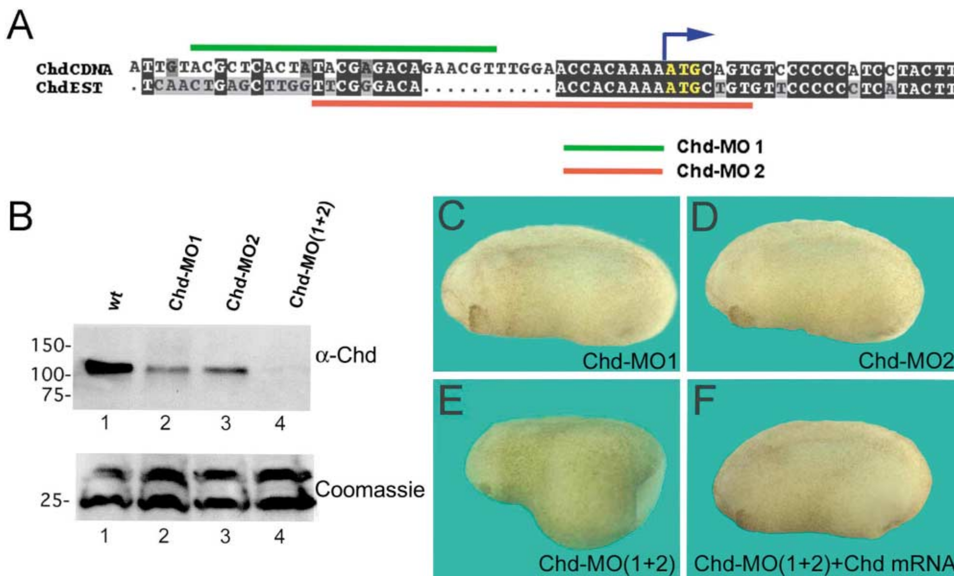

Figure 1. Morpholinos Targeting Two Distinct *chordin* mRNAs Reduce Chordin Expression and Promote Ventralization

(A) Sequence of the morpholinos targeting the two Chordin mRNAs.

(B) Western blot analysis of secreted Chordin protein. Two-cell embryos were injected four times with a total of 8 ng of Chd-MO-1 (lane 2) or Chd-MO-2 (lane 3) or with a combination of the two (4 ng each; lane 4). Dorsal lips were isolated at gastrula, and explants were cultured in Ca^{2+}-Mg^{2+}-free medium (CMFM) for 12 hr. The supernatant was then analyzed in western blots with an immunopurified anti-Chordin antibody; Coomassie blue staining of the cell pellets served as loading control.

(C–E) Embryos microinjected four times at the two-cell stage with a total of 8 ng of each Chordin morpholino or a mixture of the two. The ventralized phenotype observed by coinjection of the two morpholinos generated a highly penetrant ventralized phenotype (>75%) in many independent experiments.

(F) The effect of Chd-MO(1+2) was rescued by microinjection of 10 pg *chd* mRNA lacking the 5′ UTR.

neural plate and resulting CNS were reduced, and embryos were more susceptible to the ventralizing influence of overexpressed xTsg. Making use of the embryological manipulations available in *Xenopus*, we provide evidence that Chordin plays a central role in the dorsalization of the embryo by LiCl treatment and in the dorsalization of mesoderm by Activin protein. Transplantation of dorsal lips microinjected with morpholinos showed that Chordin is required for the inductive activity of transplanted Spemann organizer tissue.

Results

Inhibition of Both Chordin Pseudoalleles Causes Ventralization

To determine the loss-of-function phenotype of Chordin in *Xenopus* development, we designed an antisense morpholino directed against the 5′ untranslated region (UTR) of *chordin* mRNA (MO-1; Figure 1A). No phenotype was observed in a range of concentrations (Figure 1C). *Xenopus laevis* is an allotetraploid species, thought to have originated from the hybridization of two different *Xenopus* species in the course of evolution. Therefore, many genes are present in two copies, called pseudoalleles, which can differ in sequence (Kobel and Du Pasquier, 1986; Tinsley and Kobel, 1996). A search of the *Xenopus* database revealed a *chordin* EST (GenBank accession number BG019466) that differed considerably in sequence from the original *chordin* cDNA (Sasai et al., 1994). A morpholino targeting the translation initiation site of this second ortholog (Figure 1A) produced either no phenotype (Figure 1D) or weak ventralizations at high concentrations (data not shown). Analysis of the proteins secreted by dorsal lip explants with an affinity-purified anti-Chordin antibody (Larraín et al., 2001) showed that microinjection of each morpholino on its own only moderately reduced Chordin secretion but that the combination of MO-1 and MO-2 greatly downregulated production of endogenous Chordin protein (Figure 1B).

When both morpholinos, designated Chd-MO(1+2), were coinjected (4 ng of each per embryo), a ventralized phenotype was observed with high penetrance (Figure 1E). The specificity of these reagents for the two forms of Chordin was highlighted by microinjection of double the amount of MO-1 or MO-2 (8 ng per embryo), which was without phenotypic effect (Figures 1C and 1D). Embryos developed with small heads and enlarged ventroposterior regions (Figure 1E) and were reminiscent of the *chordino* phenotype in zebrafish (Hammerschmidt et al., 1996). This distinct phenotype has been previously observed in experimental conditions that lead to an increase of BMP signaling in early *Xenopus* embryos, such as the microinjection of *Xolloid*, *BMP1*, *xTsg*, *Smad5*, and *BMP4* (Piccolo et al., 1997; Goodman et al., 1998; Oelgeschläger et al., 2000; Beck et al., 2001; our unpublished data). Microinjection of a synthetic *chordin* mRNA lacking the 5′ flanking region (10 pg, once dorsal) rescued the ventralized phenotype of Chd-MO(1+2) (Figure

1F). We conclude from these experiments that Chordin morpholinos interfere with endogenous Chordin production, causing *Xenopus* embryos to develop with small heads and enlarged ventroposterior regions.

Chordin Downregulation Promotes Ventral Fates

To investigate the function of Chordin in embryonic patterning, we examined a number of molecular markers in Chd-MO(1+2)-injected embryos. The expression of *chd* and *noggin* at early gastrula, as well as the formation of the dorsal lip, were not affected (Figures 2A and 2B and data not shown). At the late gastrula stage the expression of *chd*, *noggin*, and *follistatin* mRNAs was not significantly affected by Chd-MO(1+2), except for a shortening of the length of the axial mesoderm (future notochord), presumably caused by a mild inhibition of convergence-extension gastrulation movements (Figures 2D–2F). RT-PCR analysis confirmed that the levels of *chordin*, *noggin*, or *follistatin* mRNAs were not significantly affected by Chd-MO(1+2) at early or late gastrula stages (Figure 2G). In zebrafish, the *chordino* mutation also affects the morphogenetic process of convergence extension (Myers et al., 2002). At the neurula stage, the neural plate, marked by the pan-neural marker *Sox2*, was reduced in size, particularly in the anterior (Figure 2C). The neuron-specific marker *N-tubulin* (Richter et al., 1988) confirmed the moderate reduction in CNS tissue (Figure 3E). The overall anterior-posterior patterning was only mildly affected, with a reduction of *Otx-2* in the forebrain and *HoxB9* in the spinal cord (Figure 2H). However, the ventral marker *sizzled* (which encodes a secreted Wnt antagonist of the Frzb family; Salic et al., 1997) revealed a considerable expansion of ventral tissues (Figure 2I). This is of interest because Chordin is expressed in the dorsal midline. Thus, Chordin inhibition can affect the differentiation of ventral tissue far away from its source, as it is known to be the case for its *Drosophila* homolog, Sog (Srinivasan et al., 2002).

Histological sections at the swimming tadpole stage showed that the eyes, brain, and spinal cord were reduced and that the blood islands were expanded in ventral mesoderm of Chd-MO(1+2)-injected embryos (Figures 2J–2K″). The pharynx was hypoplastic and had defects in the development of peripharyngeal structures, such as the hyoid and branchial arch cartilages (Figure 2K′ and data not shown). These pharyngeal phenotypes are reminiscent of those of the *chordin* knockout in the mouse (D. Bachiller, J. Klingensmith, and E.D.R., in preparation). The notochord was present in Chd-MO(1+2)-injected embryos (Figures 2K′, 2K″, and 3B′). This is unlike *chordino*, in which the posterior notochord is lacking, and the mouse *chordin* knockout, in which the anterior notochord regresses (Hammerschmidt et al., 1996; our unpublished data). In *Xenopus* embryos injected with Chd-MO(1+2), the myotomes were reduced and U shaped, replacing the normal chevron-shaped appearance (compare Figures 3A″ and 3B″). U-shaped somites have been described in *chordino* mutants, as well as in a large number of mutants in the Sonic hedgehog (Shh) signaling pathway in zebrafish, and reflect defects in horizontal myoseptum development caused by reduced notochord signaling. We conclude that inhibition of Chordin affects patterning of the *Xenopus* embryo, causing a reduction of dorsoanterior, and an expansion of ventroposterior, tissues.

Chd-MO(1+2) Enhances the Effect of *xTsg* mRNA

We next analyzed the effects of Chd-MO in *Xenopus* embryos overexpressing xTsg. A single ventral injection of *xTsg* mRNA caused embryos to develop with a reduced head, an expanded endodermal cavity, and a posteriorized anus, which tends to detach from the endodermal mass at the swimming tadpole stage (Chang et al., 2001; Figure 3C). This phenotype was consistent with that of a moderate increase in BMP signaling, since it was also observed in microinjections of Smad5 and constitutively active BMP receptor mRNAs (Beck et al., 2001; our unpublished data). In *xTsg*-injected embryos the notochord was hypoplastic, particularly in the posterior, and the myotomes were U shaped (Figures 3C′ and 3C″). When *xTsg* mRNA was injected into embryos in which Chordin expression was reduced by the Chd-MO(1+2), the resulting embryos were severely ventralized, with only patches of residual notochord, reduced somites, and greatly decreased numbers of N-tubulin-positive neurons in the CNS (Figures 3D and 3E). The reduction in the amount of CNS and myotomal muscle was confirmed by RT-PCR analysis (Figure 3F, lane 5).

The synergy between Chd-MO(1+2) and *xTsg* mRNA might be explained by two different mechanisms. First, xTsg is known to enhance the cleavage of Chordin by Tolloid-like proteases, both in vitro and in microinjected *Xenopus* embryos (Scott et al., 2001; Larraín et al., 2001). It is conceivable that, in our experiments, xTsg may eliminate residual amounts of Chordin protein in Chd-MO(1+2) embryos. This explanation seems unlikely, considering the *chordino* phenotype in zebrafish and the considerable reduction observed in Chordin protein levels after injection of Chd-MO(1+2) (Figure 1B). Second, a number of additional proteins that contain BMP binding cysteine-rich repeats similar to those seen in Chordin exist in vertebrates (Garcia Abreu et al., 2002); xTsg overexpresion could antagonize the activities of these other proteins as well as that of Chordin. We conclude from these results that reduction of Chordin by Chd-MO(1+2) synergizes with *xTsg* mRNA, resulting in a much stronger ventralization than that observed with either reagent on its own.

Chordin Is Required for Dorsalization by LiCl

Chordin was originally identified as a cDNA induced by lithium chloride treatment of early *Xenopus* embryos (Sasai et al., 1994). LiCl inhibits glycogen synthase kinase-3β (GSK-3), stabilizing β-catenin (Klein and Melton, 1996; Schneider et al., 1996). This, in turn, leads to the induction of *chordin* and other organizer-specific genes, such as *noggin*, *follistatin*, and *Xnr-3* (Wessely et al., 2001). Embryos treated with LiCl are dorsoanteriorized, resulting in radial head structures lacking trunk-tail structures because of the transformation of the entire marginal zone mesoderm into Spemann's organizer (Kao and Elinson, 1988; Figure 4B). As shown in Figure 4C, we found that microinjection of 200 pg *chordin* mRNA into the animal pole of four-cell *Xenopus* embryos is sufficient to mimic the LiCl phenotype (Figure 4C).

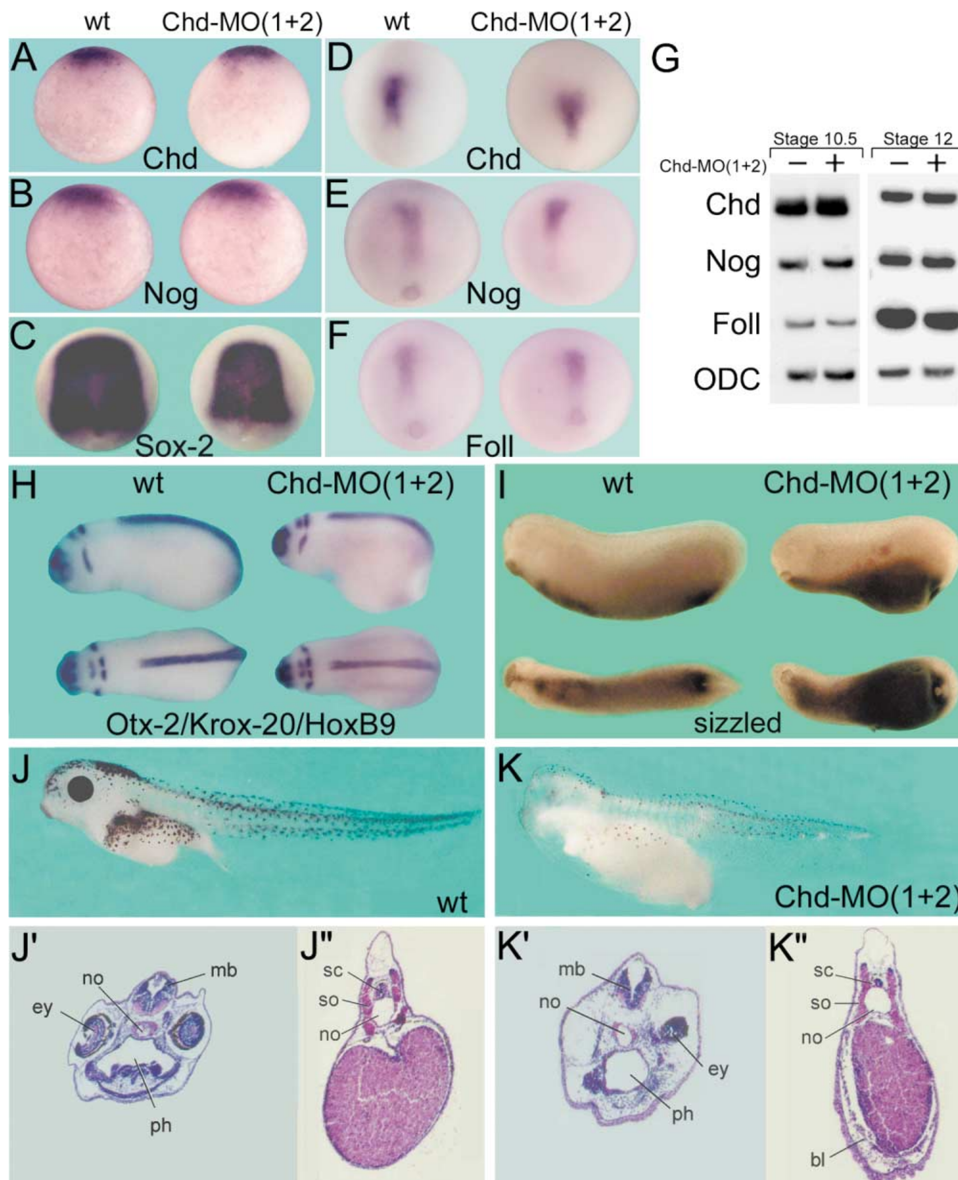

Figure 2. Inhibition of Endogenous Chordin Results in Reduction of Neuroectodermal and Expansion of Ventral Mesodermal Markers

A mixture of the two Chordin morpholinos (4 ng of each) was injected four times, radially, at the two-cell stage.

(A and B) Expression of the organizer marker genes *chordin* and *noggin* was unchanged in wild-type (wt, left) and Chd-MO(1+2)-injected (right) embryos at early gastrula.

(C) The neural plate was reduced in Chd-MO(1+2)-injected embryos, as shown by the neural marker *Sox2* (stage 12).

(D, E, and F) At stage 12 the expression of *chordin*, *noggin*, and *follistatin* was not significantly affected by Chd-MO(1+2) injection. The expression domains are shorter, indicating a mild inhibition of convergence-extension gastrulation movements.

(G) RT-PCR analysis of sibling embryos. The mRNA levels of *chordin*, *noggin*, and *follistatin* were not significantly changed in Chd-MO(1+2)-injected embryos at stage 10.5 or stage 12.

(H) At tadpole stages the A-P pattern of neural organization was relatively normal, as shown by expression of *Otx2* (forebrain), *Krox-20* (hindbrain), and *HoxB9* (spinal cord).

(I) Ventral mesoderm, marked by the expression of *sizzled*, is expanded in Chd-MO(1+2)-injected embryos. The upper panel shows a lateral view, and the lower panel shows a ventral view, of the same embryos.

(J and K) At late tadpole stages (stage 42) Chd-MO(1+2)-injected embryos develop with smaller head structures. Histological sections (J′, J″, K′, and K″) revealed a reduction of midbrain (mb), pharynx (ph), eye (ey), and spinal cord (sc) and an expansion of ventral blood islands (bl) in Ch-MO(1+2)-injected embryos. No obvious changes in somites (so) or notochord (no) were observed.

To test whether Chordin is required for the LiCl phenotype, loss-of-function experiments were performed. LiCl treatment greatly increased synthesis of Chordin protein by dissociated *Xenopus* embryos, and microinjection of Chd-MO(1+2) prior to LiCl treatment inhibited the production of secreted Chordin protein (Figure 4D). As

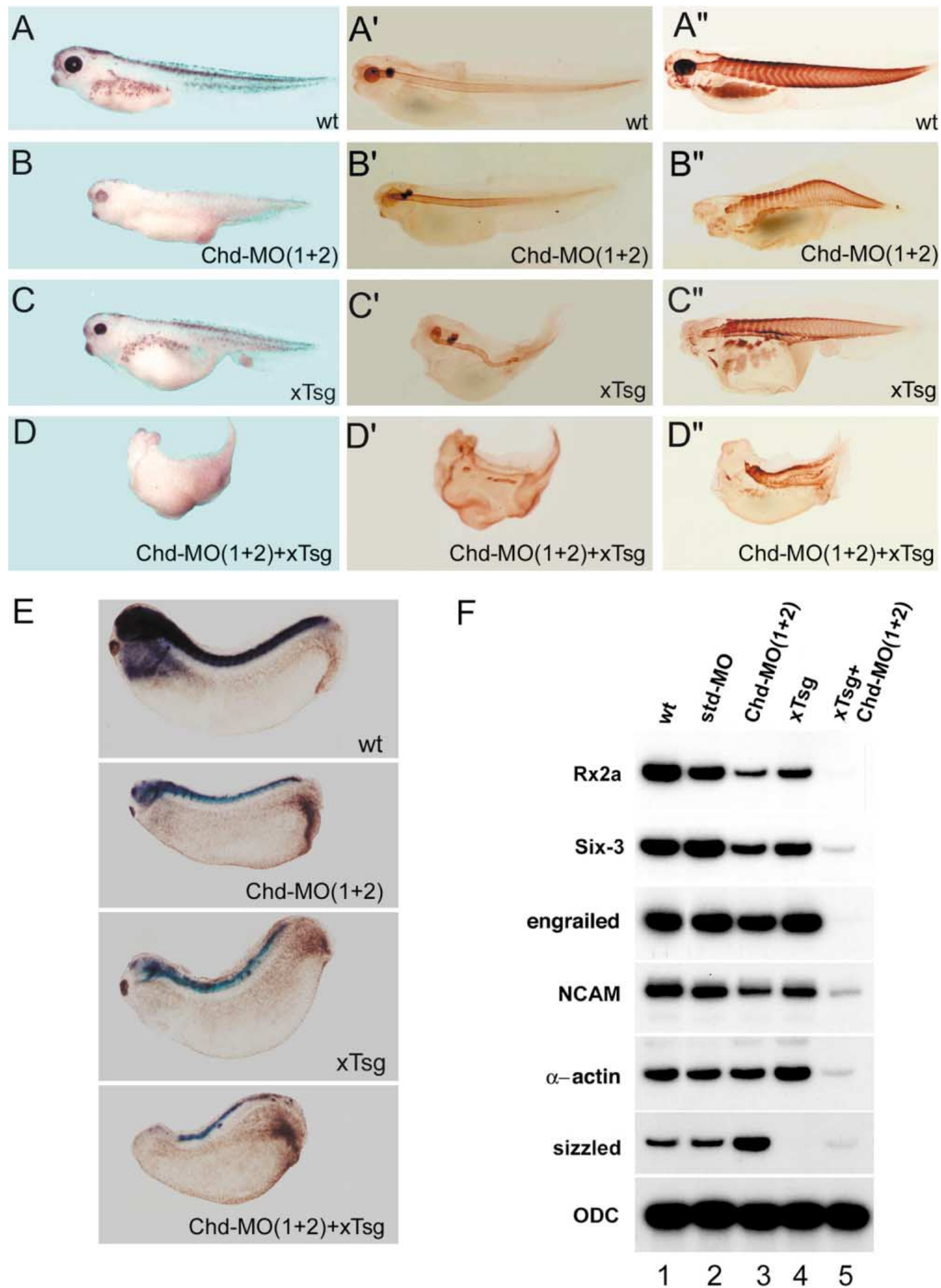

Figure 3. Reduction of Chordin and Overexpression of xTsg Synergize, Causing Ventralization of *Xenopus* Embryos

(A) Uninjected wild-type embryo at stage 40, stained with the notochord-specific antibody MZ15 (A′) or the muscle-specific antibody 12-101 (A″).

(B) Embryos microinjected with Chd-MO(1+2) at the two-cell stage have normal notochord staining (B′), but the muscles have adopted a U-shaped phenotype (B″).

(C) Embryos injected once ventrally with 1 ng of *xTsg* mRNA at the four- to eight-cell stage. Note that the notochord is reduced and absent from the posterior in strongly affected embryos (C″). Muscle development is disturbed (C″), and muscles adopt a U-shaped phenotype.

(D) Embryos coinjected with *xTsg* and Chd-MO(1+2) are strongly ventralized.

(E) *N-tubulin* expression in stage 25 wild-type embryos, embryos injected at the two-cell stage with 8 ng Chd-MO(1+2), 1 ng *xTsg* mRNA, or both.

(F) RT-PCR analysis of sibling embryos; Chd-MO(1+2) and *xTsg* mRNA cause the repression of anterior neural markers *Rx2a* (eye), *Six-3* (forebrain), and *engrailed* (midbrain). The pan-neural marker *NCAM* and the dorsal mesodermal marker *actin* are reduced. The ventral mesodermal marker *sizzled* is upregulated by Chd-MO(1+2) injections, but repressed by *xTsg* mRNA injections (Chang et al., 2001).

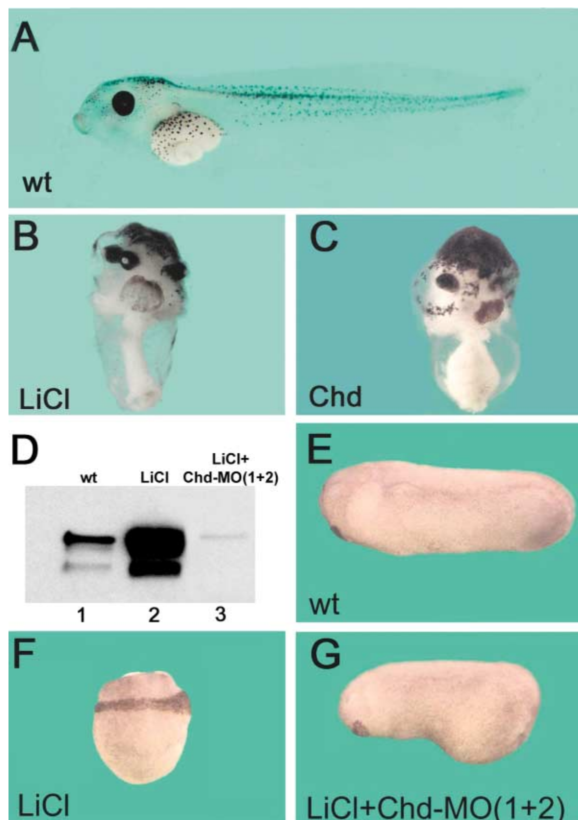

Figure 4. Chordin Is Required for the Dorsalization of *Xenopus* Embryos by LiCl Treatment

(A) Stage 45 untreated tadpole.

(B) Sibling embryo treated with 120 mM LiCl at the 32-cell stage for 30 min.

(C) Stage 45 embryo microinjected into the animal pole with 200 pg *chordin* mRNA at the four-cell stage.

(D) Chordin production is inhibited by Chd-MO(1+2), even after LiCl treatment (compare lanes 2 and 3). Whole embryos were dechorionated and dissociated at stage 9 and cultured for 14 hr at 19°C in CMFM to allow detection of Chordin protein in the supernatant.

(E and F) LiCl-treated embryos display a radial cement gland at tadpole stages and do not develop trunk-tail structures (dorso anterior index [DAI] = 8, n = 73)

(G) Embryos at the two-cell stage with 8 ng Chd-MO(1+2) before LiCl treatment are resistant to LiCl (G; DAI = 6.4, n = 38).

can be seen in Figures 4F and 4G, Chordin morpholinos blocked the effect of LiCl. In some LiCl-treated embryos the *chordino*-like phenotype with small heads and big bellies was observed (Figure 4G). Thus, although Chd-MO(1+2) had a mild effect in the intact embryo, it had a very strong effect when the embryo was experimentally dorsalized. We conclude that Chordin is required for LiCl dorsalization.

Chordin Is Required for Dorsalization of Mesoderm by Activin

At the gastrula stage the organizer secretes a "horizontal signal" that induces somite formation in ventral mesoderm (Dale and Slack, 1987). This dorsalization of the mesoderm can be mimicked by treatment of animal cap explants with the morphogen Activin (Green et al., 1992; Dyson and Gurdon, 1998). Activin has a dual activity on ectodermal animal explants. First, it is able to induce ventral mesoderm and, second, Activin generates in a dose-dependent way a dorsal-ventral set of cell fates (Green et al., 1992). Since Activin is known to induce *chordin* transcription (Sasai et al., 1994), we tested whether Chordin was required for the dorsalization of animal cap explants by Activin. Uninjected animal caps or those injected with MO-1 or MO-2 individually elongated after treatment with 2 ng/ml Activin protein (Figures 5A–5D). This elongation reflects the convergence-extension movements that take place concomitantly with dorsal mesoderm (somite) formation. When MO-1 and MO-2 were injected together, however, animal caps retained their spherical shape (Figure 5E). This effect could be rescued by the injection of *chordin* mRNA lacking the 5′ UTR (Figure 5F).

RT-PCR analysis indicated that the treatment of caps containing Chd-MO(1+2) had reduced levels of the muscle markers *Myf5* and α-*Actin* and of the neural marker *NCAM* (Figure 5G, lanes 3–5). These molecular markers were rescued by coinjection of *chd* mRNA lacking the 5′ UTR (Figure 5G, lane 6). The ability of Activin to induce ventral mesoderm was not impaired by Chordin knockdown. The ventral mesoderm markers *xHox3/eve1* and α-*Globin* were not only not inhibited, but were increased, by microinjection of Chd-MO(1+2) into Activin-treated caps (Figure 5G, lane 5).

Immunoblot analysis confirmed that 2 ng/ml Activin induced abundant secretion of Chordin protein in animal caps, which was inhibited by Chd-MO(1+2) (Figure 5H). Interestingly, when the cell pellets were analyzed with a phospho-Smad1-specific antibody, which provides a measure of BMP receptor activity (Persson et al., 1998), Activin repressed, and Chd-MO(1+2) restored, BMP signaling (Figure 5H). The induction of the BMP antagonists *noggin*, *follistatin*, and *cerberus* by Activin treatment in animal cap explants was not significantly affected by Chd-MO(1+2) injection (Figure 5I). At the end of gastrulation, at a time by which competence to respond to Activin is lost (Grimm and Gurdon, 2002), *noggin* and *follistatin* transcription was only weakly reduced, whereas the levels of *chordin* transcripts were clearly downregulated by Chd-MO(1+2) (Figure 5J, lane 4). Taken together, the results suggest that Chordin plays a critical role in the dorsalization of mesoderm by Activin treatment but is not required for the induction of ventral mesoderm by this morphogen.

Chordin Is Required for Spemann Organizer Activity

To further analyze the function of Chordin in dorsal development, we carried out two series of transplantation experiments. Dorsal lips were isolated from early gastrulae microinjected with the lineage tracer biotin dextran amine (BDA) with or without Chd-MO(1+2). The graft tissue comprised 30° of the marginal zone and extended from the base of the blastocoel to the early dorsal lip. In the first experimental series (Figure 6A), the dorsal lip replaced the endogenous organizer (isotopic and isochronic transplantation). In the absence of morpholinos the graft contributed to axial structures, in particular, to the notochord and dorsal endoderm, and noninvoluted cells contributed to ventral CNS, including the floor

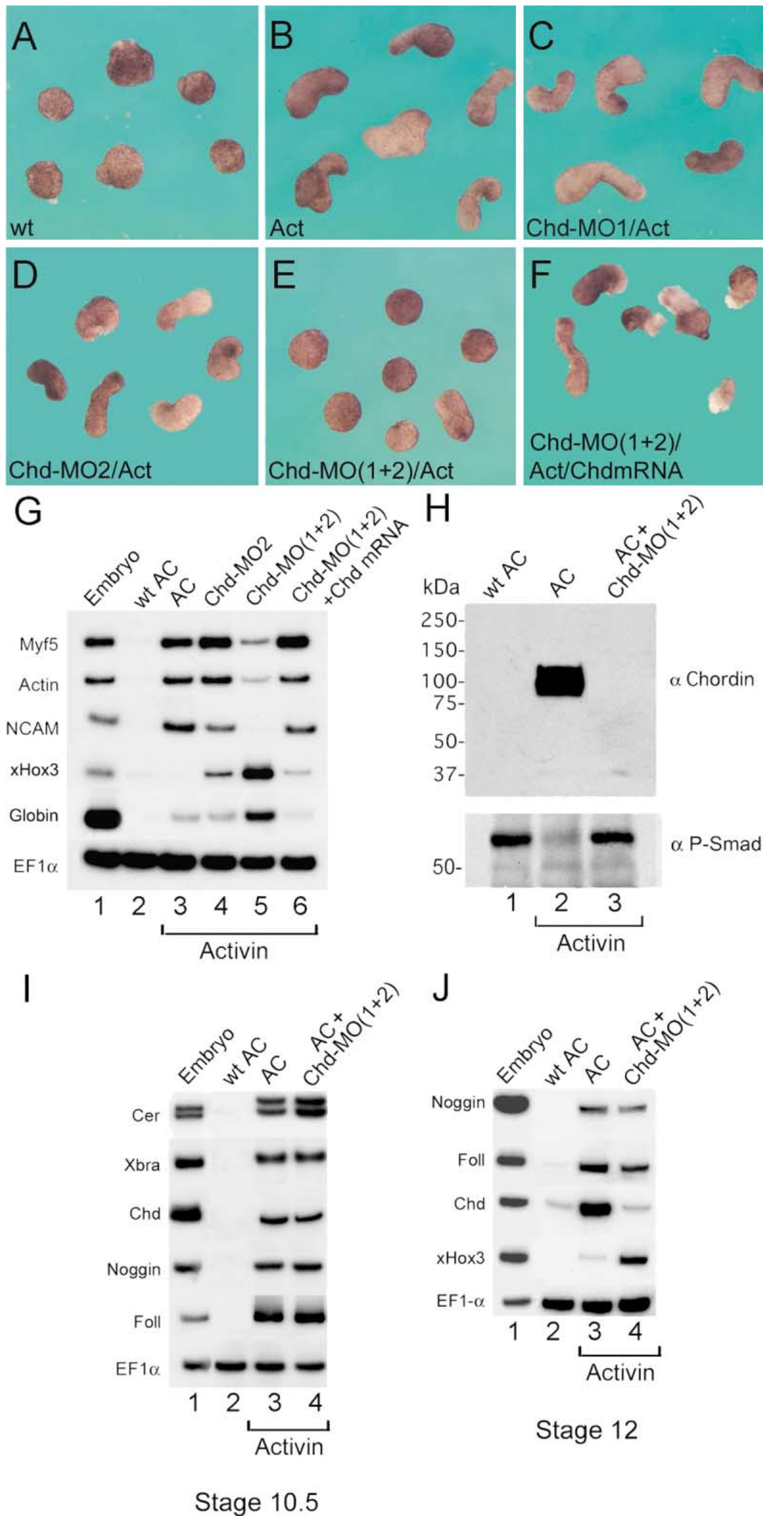

Figure 5. Chordin Is Required for the Induction of Dorsal Mesoderm by Activin

(A–F) Two-cell embryos were injected four times into the animal pole with a total of 8 ng of Chd-MO-1, Chd-MO-2, Chd-MO(1+2), or with Chd-MO(1+2) and 10 pg of *chordin* mRNA lacking the 5′ UTR of the *chordin* cDNA. Animal explants were isolated at stage 8, treated with 2 ng/ml Activin, and cultured until stage 25. Three independent experiments were performed with similar results.

(G) RT-PCR analysis of samples shown in (A–F). Activin treatment induced the expression of the somite marker genes *Myf5* and *Actin* and the pan-neural marker *NCAM*. Chd-MO(1+2) inhibited the expression of dorsal markers but upregulated the expression of ventral marker genes (*xHox3* and α-*Globin*).

(H) A subset of the animal caps were cultured overnight in CMFM. The supernatant was analyzed in Western blots with an immunopurified antibody specific for the interrepeat region of Chordin (α-I-Chd; Larraín et al., 2001), and the cell pellet was analyzed with an antibody specific for phospho-Smad-1.

(I) RT-PCR analysis of Activin-treated animal caps at stage 10.5 injected with Chd-MO(1+2). The morpholinos did not affect mesoderm induction (*Xbra*) or the expression of other BMP antagonists (*cerberus*, *chordin*, *noggin*, and *follistatin*).

(J) RT-PCR analysis of Activin-treated animal caps at stage 12. The microinjection of Chd-MO(1+2) changed *noggin* and *follistatin* mRNA levels only slightly, inhibited *chordin* expression, and upregulated the ventral mesoderm marker *xHox3*.

plate (Figure 6B). Transplanted organizers microinjected with Chd-MO(1+2) involuted and contributed normally to dorsal endoderm and notochord, but, in addition, labeled cells were found in somites (Figure 6C). The cells in the somites were found in the medial region adjacent to the notochord (Figure 6C) and indicate a modest ventralization of the mesoderm.

Unexpectedly, we noted that, in Chd-MO(1+2)-injected organizers, the noninvoluted cells no longer contributed to the CNS or floor plate but, rather, were found in the epidermis, particularly in the posterior region of the embryo (Figure 6C). This phenotype was fully penetrant, with all serially sectioned morpholino-injected transplants showing staining in the epidermis,

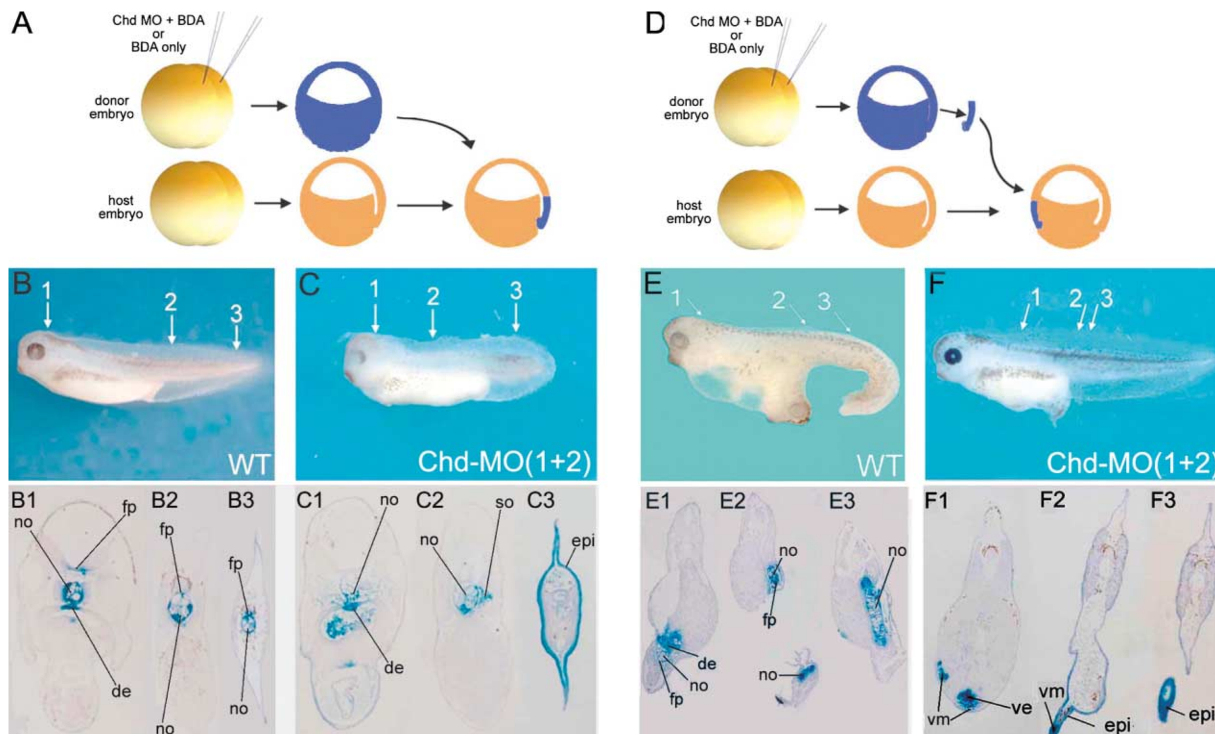

Figure 6. Chordin Is Required for Spemann's Organizer Activity

(A) Experimental design of the isotopic and isochronic transplantation experiment. Embryos were injected twice into dorsal blastomeres with BDA (biotin dextran amine lineage tracer) alone or with Chd-MO(1+2). Dorsal lip explants were isolated from these donor embryos and transplanted at gastrula into unlabeled host embryos. After the embryos were sectioned in paraffin wax, BDA was detected with streptavidin-conjugated alkaline phosphatase and BM purple.

(B) Transplantation of an organizer injected with lineage tracer alone allowed normal development in most cases, but some embryos developed shortened axes (12%, n = 33). Histological sections showed that BDA staining was detected in the notochord (no) and floor plate (fp) throughout the embryo and in anterior dorsal endoderm (de). Seven embryos were serially sectioned, stained with Streptavidin-AP, and developed with BMP purple.

(C) Embryos that received Chd-MO(1+2)-injected organizer transplants developed with shortened body axes (100%, n = 15) and small eyes (47%, n = 15) or no eyes (20%, n = 15). Lineage label was detected in the notochord, anterior dorsal endoderm, and medial somite (so). Surprisingly, transplanted cells stained the epidermis of the host embryo (epi) in the posterior and were excluded from neural tissue. Six embryos were serially sectioned.

(D) Dorsal lip explants transplanted into the ventral side of a host embryo recapitulate the classical Spemann-Mangold experiment.

(E) The transplantation of a wild-type organizer induced the formation of secondary axes (100%, n = 17), of which most (82%) were complete axes containing heads and eyes. In the 11 embryos serially sectioned, BDA could be detected in the notochord, floor plate, and dorsal endoderm of the secondary axes.

(F) Transplantation of a Chd-MO(1+2)-injected organizer blocked the axis-inducing activity of the explant. A few embryos formed protrusions (31%, n = 16), the strongest of which is shown here. In the seven embryos serially sectioned, BDA staining was detected in the ventral mesoderm (vm), ventral endoderm (ve), and epidermis (epi). Note that Chd-MO(1+2)-injected grafts did not induce the CNS, somites, or notochord.

whereas all sectioned control grafts showed labeling in the involuted endomesoderm and CNS, but not in epidermis. This surprising result suggests that Chordin is required in a cell-autonomous way in the ectoderm for differentiation of ectodermal cells into CNS. The embryos transplanted in this way do form a ventral CNS but, when given a choice in this chimeric situation between Chordin-expressing and -nonexpressing ectodermal cells, prefer the Chordin-expressing cells. At the blastula stage *chordin* is expressed in the preorganizer region located in the dorsal animal cap, which might contribute to the future neural plate (Wessely et al., 2001).

In the second series of experiments, the dorsal lip was transplanted ventrally, as in the classical Spemann-Mangold experiment (Figure 6D). In the controls, secondary axes were induced at high frequency (n = 17) and, in most cases, were complete and included eyes, with the grafted cells contributing to notochord, floor plate, and dorsal endoderm (Figure 6E). Microinjection of Chd-MO(1+2) had a striking effect on the graft, abolishing secondary axis induction (n = 16). In some embryos weak ectodermal protrusions were seen, and the strongest one is shown in Figure 6F. Chd-MO(1+2)-treated organizers were completely unable to induce CNS, somites, notochord, or secondary gut. The grafted cells contributed mostly to ventral mesesoderm, endoderm, and epidermis. In none of the serially sectioned control Spemann grafts was epidermal staining observed, whereas all Chd-MO(1+2) grafts analyzed had labeled cells in the epidermis.

Figure 7 shows the differentiation of dorsal lips cultured for three days in saline solution (stage 38). Dorsal

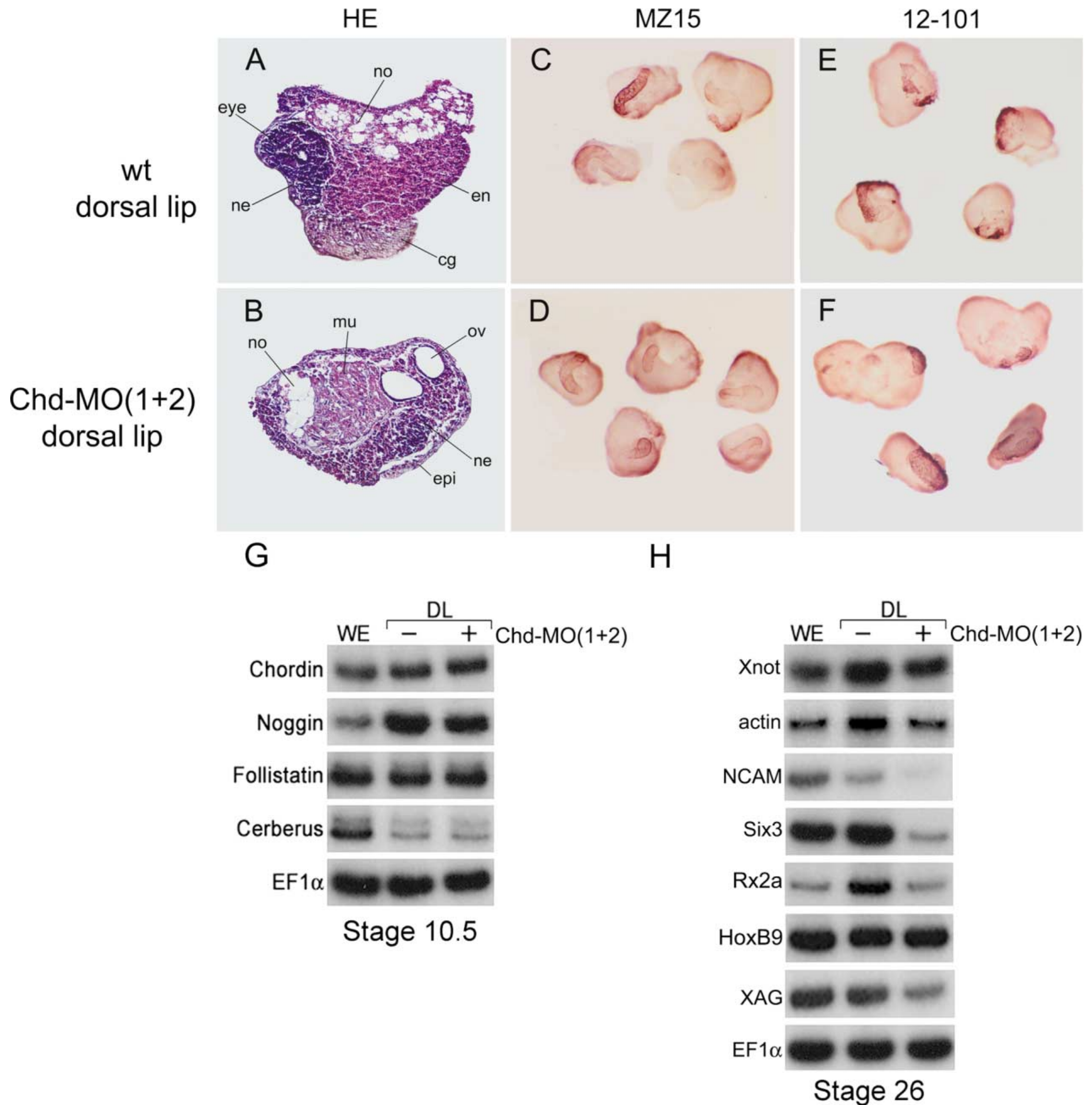

Figure 7. The Mesodermal Specification of Dorsal Lip Explants Is Not Changed by Chd-MO(1+2) Microinjection

(A) Explanted dorsal lips (n = 25) developed into anterior neural tissue (eye, ne), notochord (no), endoderm (en), and cement gland (cg) at stage 38.

(B) Chd-MO(1+2)-injected dorsal lip explants (n = 27) developed into notochord (no), muscle (mu), posterior neural tissue (ne), otic vesicle (ov), and epidermis (epi).

(C and D) Uninjected and Chd-MO(1+2)-injected dorsal lip explants were positive for the notochord-specific antibody MZ15 (n = 5).

(E and F) Uninjected and Chd-MO(1+2)-injected dorsal lip explants were stained by the muscle-specific antibody 12-101.

(G) RT-PCR analysis of dorsal lip explants at stage 10.5 showing that organizer gene expression (*chd*, *noggin*, *follistatin*, and *cerberus*) was not affected by Chd-MO(1+2) at the time of the transplantations.

(H) RT-PCR analysis of Chd-MO(1+2)-injected dorsal lip explants at stage 26. In Chd-MO(1+2)-microinjected embryos the anterior neural markers *Six-3* and *Rx2a* were inhibited, the pan-neural marker *NCAM* and the cement gland marker *XAG* were reduced, and the notochord marker *Xnot* and the somite marker gene *actin* were not affected.

lip explants identical to those used for the grafting experiments differentiate into anterior brain (containing eye tissue), notochord, cement gland, and some muscle (Figure 7A; n = 25). The organizer retained its specification as dorsal mesoderm in Chd-MO(1+2)-injected explants, forming notochord and muscle (Figure 7B; n = 27). Antibody markers confirmed that notochord (Figure 7D; 5 out of 6 explants) and muscle (Figure 7F; 5 out of 5 explants) were present in Chd-MO(1+2)-injected dorsal lips. The expression of organizer-specific BMP antagonists was not affected by Chd-MO(1+2) in dorsal lips analyzed by RT-PCR at the time of transplantation

(Figure 7G). Whereas the specification of mesoderm was not changed, the neural tissue was reduced and had a more posterior character in Chd-MO(1+2)-injected dorsal lips (Figure 7B). Uninjected dorsal lip explants contain cement glands but, as is well known, are not surrounded by epidermis (Holtfreter, 1938; Figure 7A). Morpholino-injected dorsal lips had smaller cement glands and were covered by epidermis in nonneural regions (Figure 7B), indicating ectoderm of a more ventral character. Molecular analyses of dorsal lips cultured until tailbud stages (Figure 7H) confirmed that dorsal mesoderm specification was maintained. Since the specification of the gastrula mesodermal organizer is unchanged, these transplantation experiments indicate that Chordin protein is required for the inductive properties of Spemann organizer tissue transplanted into the ventral side of *Xenopus* embryos.

Discussion

Spemann's organizer has been studied intensively in Amphibia, but loss-of-function studies have not been possible. Using antisense morpholino oligomers we now show that the phenotype of Chordin knockdown in whole *Xenopus* embryos resembles that of *chordino* mutants in zebrafish. Considering the similarities between *Xenopus* and zebrafish development, this result is not remarkable in itself. However, when embryonic development was challenged in a number of experimental situations, an unexpectedly strong requirement for Chordin was revealed. In particular, we found that dorsalization by LiCl, Activin, and Spemann organizer grafts was dependent on Chordin function. The loss of one gene product may be compensated by redundant molecular mechanisms in the context of the whole embryo. In experimental conditions, compensatory mechanisms may be insufficient to mask the effects of the loss of Chordin protein. The results illustrate the importance of being able to manipulate the embryo in order to understand molecular mechanisms of embryonic induction in vertebrates.

Chordin knockdown phenocopies the *chordino* mutation in zebrafish (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). The *Xenopus* embryos displayed a moderately ventralized phenotype, with reduced head and expanded ventroposterior structures. Overall, the effect in intact embryos was relatively mild, and neural tissue was induced, although the neural plate was somewhat reduced (Figure 2). This weak ventralized phenotype was greatly enhanced in embryos injected with *xTsg* mRNA (Figure 3). Vertebrates have many Chordin-like CR-containing proteins, and xTsg can interact with several of them (Garcia Abreu et al., 2002; our unpublished data). It is possible that these or other redundant molecular mechanisms, such as those of the BMP antagonists Noggin, Follistatin, Xnr3, or Cerberus, might compensate for the lack of Chordin in whole embryos. However, this compensation does not take place at the transcriptional level, at least for *noggin* and *follistatin*, in Chd-MO(1+2)-injected embryos (Figure 2G). In the mouse the *noggin* and *chordin* gene products can compensate for each other, since their requirement for forebrain development is only seen in the double mutant (Bachiller et al., 2000). In *Xenopus*, an embryo that can be easily manipulated, it is possible to uncover the requirement of Chordin for embryonic induction with a variety of experimental treatments.

Spemann's organizer is induced by an early nuclear β-catenin signal on the dorsal side of the embryo (reviewed in Harland and Gerhart, 1997; De Robertis et al., 2000). Treatment of embryos with LiCl at the 32-cell stage stabilizes β-catenin, leading to the formation of a radial organizer spanning the entire marginal zone (Kao and Elinson, 1988). LiCl embryos develop as radial head structures lacking trunk-tail structures, which can be phenocopied by overexpression of Chordin in the animal pole (Figure 4C). Chordin is greatly upregulated by LiCl treatment (Wessely et al., 2001), and, remarkably, the phenotypic effects of LiCl can be completely blocked by Chordin morpholinos (Figures 4F and 4G). Rescue of LiCl dorsalization has been previously obtained by treatments that increase BMP signaling, such as microinjection of *BMP4*, *Xolloid*, or *xTsg* mRNAs (Fainsod et al. 1994; Larraín et al., 2001). The present results indicate that the transcriptional upregulation of the BMP antagonist Chordin is an essential downstream component mediating the effect of LiCl on embryonic patterning.

Another agent that induces Chordin is the TGF-β superfamily member Activin. In animal cap explants strong induction of Chordin protein by Activin was observed and was blocked by Chd-MO(1+2). Activin treatment reduces Smad1 phosphorylation (a readout of BMP signaling), which was restored in the presence of Chd-MO(1+2) (Figure 5H). Animal cap elongation, muscle differentiation, and neural differentiation were inhibited by Chd-MO(1+2). Thus, Chordin appears to be required for the dorsalization of ventral mesoderm by Activin, a much-studied morphogen (Green et al., 1992; Dyson and Gurdon, 1998). However, the induction of ventral mesoderm by Activin is not inhibited by Chd-MO(1+2). The transcription of *noggin* and *follistatin* was not significantly affected in these experiments. In the future it will be interesting to analyze the effects of Chordin protein level reduction on the dose-dependent threshold effects of Activin and endogenous Xnr signals on mesoderm patterning.

Overexpression of BMP4 antagonizes the induction of dorsal mesoderm by Activin (Jones et al., 1992). In previous work, Goodman et al. (1998) showed that the metalloproteinase Xolloid or BMP1 blocked muscle formation in Activin-treated animal caps. These enzymes cleave Chordin as well as other substrates, such as probiglycan, procollagen, and prolysyl oxidase (Scott et al., 2000; Uzel et al., 2001); the present work demonstrates that Chordin is the component required for the Xolloid/BMP1 effect.

In addition to the dorsalization of ventral mesoderm by LiCl and Activin, Chordin is required for the inductive activity of dorsal lips transplanted into the ventral side of a host gastrula. Chd-MO(1+2)-injected dorsal lips were completely unable to induce CNS, somites, or notochord, and they themselves differentiated into ventral endoderm, mesoderm, and ectoderm (Figure 6F). This result is in sharp contrast to the phenotype seen in intact embryos, in which dorsal tissues continue to differentiate after microinjection of Chd-MO(1+2). It seems possible that, in whole embryos, other BMP antagonists can

compensate for the lack of Chordin, but not when a small group of cells is introduced into ventral tissue and presented with the challenge of transforming ventral cells expressing high BMP levels into dorsal tissue at the gastrula stage. In Spemann grafts the lack of a single BMP antagonist, Chordin, has a profound effect on neural-inducing activity and on the self-differentiation of the graft. Interestingly, mouse node transplants have been shown to be impaired in their neural-inducing activity after transplantation into chick hosts when the organizer gene goosecoid is mutated, even though neural induction is normal in intact $gsc^{-/-}$ mouse embryos (Zhu et al., 1999). One possibility is that, since BMP expression is under positive feedback (Hammerschmidt et al., 1996), the grafted tissue is overrun by BMP from surrounding ventral tissue when Chordin is lacking. Another possibility is that the mild phenotype observed in whole embryos may result from organizer-independent dorsalization signals, such as, for example, FGFs or Xnr's. The present experiments address only the loss-of-function of Chordin. Other BMP antagonists, such as Noggin, Follistatin, and Cerberus, could also be required for the Spemann organizer phenomenon. In the future it will be interesting to test these individually.

An intriguing result noted in our transplantation experiments was that, in organizers transplanted into the dorsal side (Figure 6A), ectodermal cells were unable to contribute to the CNS and floor plate when injected with Chd-MO(1+2), forming only epidermis. Thus, it appears that Chordin is required in a cell-autonomous way in the ectoderm for the differentiation of neural plate in these transplantation experiments. Recently, Baker et al. (1999) showed that β-catenin overexpressed in the ectoderm greatly expands the neural plate, and Wessely et al. (2001) described a β-catenin-dependent region of *chordin* expression in the preorganizer region located in the dorsal side of the animal cap shortly after midblastula. Future studies will address whether the early expression of *chordin* in the blastula preorganizer is required for neural induction.

Experimental Procedures

Morpholino Oligomers

Morpholino antisense oligomers were obtained from Gene Tools. Chd-MO1 had the sequence 5′-ACGTTCTGTCTCGTATAGTGAG CGT-3′, Chd-MO2 had the sequence 5′-ACAGCATTTTTGTGGTTG TCCCGAA-3′, and the standard morpholino that was used as a negative control had the sequence 5′-CCTCTTACCTCAGTTACAA TTTATA-3′. The morpholinos were resuspended in sterile water to a concentration of 1 mM and injected either four times into the animal pole or twice into the dorsal side of two-cell embryos. Injections at later stages reduced the penetrance of the phenotype.

Embryological Methods

Microinjections, whole-mount in situ hybridization, immunocytochemistry, and mRNA synthesis were performed as described (Piccolo et al., 1997; Oelgeschläger et al., 2000; Sive et al., 2000). RT-PCR and the biotin dextran amine (BDA) lineage-tracing method are described elsewhere (Sasai et al., 1995; Bouwmeester et al., 1996; http://www.hhmi.ucla.edu/derobertis/index.html). For LiCl treatment the embryos were incubated for 25 min with 120 mM LiCl in 0.1 × MBS at the 32-cell stage, and the dorsoanterior index (DAI) was determined after stage 28 (Kao and Elinson, 1988).

Protein Analyses

Chordin protein secreted from either dorsal lip explants or Activin-treated animal caps was analyzed after a 12 hr incubation of ten explants for each sample in 50 μl CMFM at room temperature. The supernatant was directly used for Western blot analysis with an antibody specific for the interrepeat region of Chordin (α-I-Chd; Piccolo et al., 1997; Larraín et al., 2001) that had been blot affinity purified (Larraín et al., 2001). For the analysis of phospho-Smad1 levels in *Xenopus* explants, cells were homogenized in lysis buffer (Faure et al., 2000). Anti-phospho-hSmad1 antibody (Persson et al., 1998) was used at a 1:6000 dilution for Western blots.

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