Xenopus Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis

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SUMMARY

During amphibian development, non-canonical Wnt signals regulate the polarity of intercalating dorsal mesoderm cells during convergent extension. Cells of the overlying posterior neural ectoderm engage in similar morphogenetic cell movements. Important differences have been discerned in the cell behaviors associated with neural and mesodermal cell intercalation, raising the possibility that different mechanisms may control intercalations in these two tissues. In this report, targeted expression of mutants of *Xenopus Dishevelled* (*Xdsh*) to neural or mesodermal tissues elicited different defects that were consistent with inhibition of either neural or mesodermal convergent extension. Expression of mutant *Xdsh* also inhibited elongation of neural tissues in vitro in Keller sandwich explants and in vivo in neural plate grafts. Targeted expression of other Wnt signaling antagonists also inhibited neural convergent extension in whole embryos. In situ hybridization indicated that these defects were not due to changes in cell fate. Examination of embryonic phenotypes after inhibition of convergent extension in different tissues reveals a primary role for mesodermal convergent extension in axial elongation, and a role for neural convergent extension as an equalizing force to produce a straight axis. This study demonstrates that non-canonical Wnt signaling is a common mechanism controlling convergent extension in two very different tissues in the *Xenopus* embryo and may reflect a general conservation of control mechanisms in vertebrate convergent extension.

Key words: Dishevelled, Wnt, Convergent extension, Neural tube defects, *Xenopus*

INTRODUCTION

The formation of the vertebrate nervous system is a complicated developmental process that requires a tightly coordinated suite of cell-cell interactions, intracellular signaling events, gene transcription, and morphogenetic cell movements. Significant strides have been made in understanding the molecular control of induction and cell fate specification in the neural tube (Harland, 2000). Recent progress has also been made in understanding the cellular mechanisms by which neural morphogenesis occurs (Smith and Schoenwolf, 1997). One morphogenetic process that occurs in the amphibian nervous system is the narrowing and lengthening of the posterior neural tissues (Burnside and Jacobson, 1968; Jacobson and Gordon, 1976; Keller et al., 1992b; Keller, 1975). The presumptive posterior hindbrain and spinal cord undergo convergent extension movements in parallel with similar movements which occur in the underlying mesoderm (Fig. 1A). Despite the proximity of the two tissues and the parallel nature of the movements, neural convergent extension is not a passive event, but rather an active, autonomous morphogenetic process that results from intercalation of neural ectodermal cells (Elul and Keller, 2000; Elul et al., 1997; Keller and Danilchik, 1988; Keller et al., 1992b).

The cellular mechanics that underlie convergent extension are best understood in the dorsal mesoderm of *Xenopus* embryos (Keller et al., 1992a). The process is driven by mediolaterally oriented, bipolar protrusions that exert traction on neighboring cells resulting in mediolateral intercalation (Fig. 1B; Shih and Keller, 1992a; Shih and Keller, 1992b). Disruption of the stability or polarity of this protrusive activity inhibits convergent extension (Wallingford et al., 2000). Convergent extension of the posterior neural plate involves quite different cell behaviors. Intercalation of more lateral cells in the posterior neural plate involves medially oriented, monopolar protrusions, whereas the more medial cells of the notoplate display randomly oriented motility (Fig. 1C; Elul and Keller, 2000). These very different cell behaviors suggest that the molecular control of convergent extension in mesodermal and neural tissues may be quite distinct.

Curiously, when posterior neural tissue is cultured in isolation, with no apposed mesoderm, neural cells will intercalate using bipolar, mediolaterally oriented lamellipodia (Fig. 1D; Elul et al., 1997). It has been suggested that this bipolar mode of intercalation represents a latent control mechanism in the neural plate (Keller et al., 2000), raising the possibility that a common mechanism underlies both neural and mesodermal convergent extension, but that additional

levels of regulation in the two germ layers account for the differences in cell behaviors.

Little is known about the molecular control of convergent extension in any tissue, but members of the Wnt signal transduction network have been implicated in controlling this process in the *Xenopus* dorsal mesoderm. Indeed, Wnt11, Wnt5a, Frizzled-8 (*Xfz-8*), Frizzled-7 (*Xfz-7*), Naked Cuticle and Dishevelled (*Xdsh*) have each been shown to modulate convergent extension in dorsal mesoderm (Deardorff et al., 1998; Medina et al., 2000; Moon et al., 1993; Sokol, 1996; Tada and Smith, 2000; Yan et al., 2001). A variety of studies have indicated that these Wnt signaling components control convergent extension in *Xenopus* mesoderm via non-canonical Wnt pathways. For example, experiments using deletions of Xdsh that selectively affect the canonical or non-canonical Wnt pathways indicate that control of convergent extension occurs via a vertebrate cognate of the *Drosophila* planar cell polarity cascade (Tada and Smith, 2000; Wallingford et al., 2000). Likewise, defects in mesodermal convergent extension elicited by expression of interfering mutants of *Xfz-7* or *Xfz-8* can be rescued by co-expression of Xdsh, but not by other molecules that specifically activate the canonical Wnt pathway (Djiane et al., 2000; Wallingford et al., 2001b). Consistent with a role for planar polarity signaling, time-lapse analysis of cell behaviors has also revealed that Xdsh controls the polarity of the lamellipodial protrusions that drive convergent extension in the dorsal mesoderm (Wallingford et al., 2000).

In this report, we used interfering mutants of Wnt signaling components to address the possibility that a common mechanism regulates convergent extension in both neural and mesodermal cells, despite the tremendous differences in cell fates between the two tissues. Using targeted injections, explants and grafting, we have shown that mutants of *Xdsh* that can inhibit mesodermal convergent extension also directly inhibit neural convergent extension, indicating that a common, Xdsh-mediated control mechanism lies at the root of both processes. These findings reflect a general conservation of regulatory mechanisms in vertebrate convergent extension. Finally, these experiments highlight the relative contribution of both neural and mesodermal convergent extension to the coordinated elongation of the anteroposterior axis.

MATERIALS AND METHODS

Embryos, microinjection, in situ hybridization and constructs

Female adult *Xenopus laevis* were ovulated by injection of human chorionic gonadotropin and eggs were fertilized in vitro, dejellied in 3% cysteine (pH 7.9) and subsequently reared in 1/3× MMR. For microinjections, embryos were placed in a solution of 2.5% ficoll in 1/3× MMR, and injected using an Oxford universal micromanipulator into the dorsal-animal or dorsal-vegetal two blastomeres at the eight-cell stage, reared in ficoll + 1/3×MMR to stage 9, then washed and reared in 1/3× MMR alone. Embryos were fixed in MEMFA at stage 30. Embryos were staged according to (Nieuwkoop and Faber, 1994). Embryo culture, microinjection, solutions used, in vitro transcription, in situ hybridization and antibody staining were as previously described (Sive et al., 2000). Constructs used were Xdsh-ΔPDZ/D2 (Xdsh-D2) and Xdsh-ΔDEP/D6 (Rothbächer et al., 2000), Xdd1 (Sokol, 1996), XBF-2 (Mariani and Harland, 1998), Xwnt5a (Moon et al., 1993), and Nxfz-8 (Deardorff et al., 1998).

Keller sandwiches

To assess neural convergent extension directly, embryos were injected dorsally and animally at the four-cell stage and reared to gastrula stages as described above. At stage 10.5, 60° dorsal marginal zone explants were cut, centered on the midline of the dorsal blastopore lip. Dissections were made using eyebrow knives and forceps. Two explants were cut to the same size and sandwiched together (see Fig. 4A) under a raised coverslip until the sandwich had healed (about 1 hour); the coverslip was then removed and explants were cultured in agarose-coated dishes. All explants were cut and reared in 1× Steinberg's solution. Convergent extension was assessed when unmanipulated sibling control embryos had reached stage 20.

Animal cap explants

For animal cap experiments, embryos were injected as above, but into the animal pole of one-cell embryos and cultured until stage 9 in 1/3× MMR. Animal caps were removed using forceps at stage 9 and cultured to stage 20 in individual agarose wells. Animal caps were cut and reared in 3/4 NAM.

Neural plate grafts and ablations

To assess neural convergent extension in vivo, heterochronic neural plate grafts were prepared as described (Mariani et al., 2001; Ribisi et al., 2000). RNA encoding either GFP or Xdsh-D2 was injected into the animal pole of both cells of two-cell embryos. Injected embryos were reared at 12° C overnight and sibling uninjected embryos were reared at 15° C. When uninjected embryos reached stage 11.5, the posterior neural ectoderm was removed using forceps and an eyebrow knife with embryos in agarose wells. For ablations, operated embryos were cultured in 1/3× MMR until tadpole stages and fixed in MEMFA. For grafts, an animal cap was removed from a stage 9 injected embryo and heterochronic grafts were made into the uninjected dissected embryo (see Fig. 8A). For healing, host and graft were transferred to a clay-bottomed dish, the graft was held in place on the host by coverglass supported with modeling clay, and cultured in 1× Steinberg's until it had healed (roughly 30 minutes), then transferred to agarose-coated dishes with 1/3× MMR and reared to tadpole stages. For mock operations, the neural plate was cut on three sides, separated from the underlying mesoderm and then replaced. Only embryos that finished gastrulation normally and closed their blastopores were analyzed. Notochords were measured using NIH image.

RESULTS

Targeted expression of mutant Xdsh reveals a new convergent extension phenotype

Fate maps of the eight-cell *Xenopus* embryo (Moody and Kline, 1990) indicate that neural ectoderm derives predominantly from the dorsal animal blastomeres, while dorsal mesoderm derives from both animal and vegetal dorsal blastomeres (Fig. 2A). To test the idea that Xdsh may regulate convergent extension movements in the neural tube, we biased expression of a mutant of *Xdsh* to the presumptive neural tube by microinjecting mRNA into the dorsal animal blastomeres at the eight-cell stage and examined the resulting phenotype. Because canonical Wnt signaling may be involved in cell fate determination in the neural tube (Baker et al., 1999; McGrew et al., 1997; McGrew et al., 1995), we tested the effects of Xdsh-ΔPDZ/D2 (Xdsh-D2), which is a potent inhibitor of convergent extension in *Xenopus* mesoderm (Wallingford et al., 2000) but does not inhibit canonical Wnt signaling (Rothbächer et al., 2000).

When Xdsh-D2 was injected in the dorsal animal

blastomeres at the eight-cell stage, the majority of embryos (54%, n=123) failed to close their neural tubes by stage 20 (Fig. 2C, arrow), and most others displayed obvious defects in fusion of the neural folds (Fig. 2C, arrowhead). At stage 30, these embryos displayed a severe dorsal flexure and a failure to elongate the anteroposterior (A/P) axis (Fig. 2F), though anterior structures were unaffected. This phenotype is similar to that reported for dorsal injection of another mutant of Xdsh, Xdd1, at the four-cell stage (Sokol, 1996). Similar phenotypes are also observed after dorsal injection of mutant or wild-type *Xfz-8* or *Xfz-7* (Deardorff et al., 1998; Djiane et al., 2000; Medina et al., 2000; Wallingford et al., 2001b)

Surprisingly, when injection of Xdsh-D2 was biased to the mesoderm by injection into the dorsal vegetal blastomeres at the eight-cell stage, a strikingly different phenotype was observed. When embryos reached stage 20, only a very few (12%, n=89) displayed failure of neural tube closure, and a few embryos had subtle defects in the fusion of the neural folds (Fig. 2D, arrowhead). The majority closed their neural tubes normally. When these embryos developed to stage 30, almost none displayed the prominent dorsal flexure that was characteristic of embryos injected animally at the eight-cell stage or dorsally at the four-cell stage. Instead, the A/P axes of these embryos remained straight, but were very severely shortened and somewhat wider compared with control embryos (Fig. 2G). Again, anterior structures appeared normal. This morphology is distinctly different from those injected in the dorsal animal blastomeres (Fig. 2F).

Identical results were also obtained with targeted injections of the Xdsh-ΔDEP (D6) mutant (data not shown), which (like Xdsh-D2) is functional for canonical Wnt signaling (Rothbächer et al., 2000) and inhibits mesodermal convergent extension (Wallingford et al., 2000).

The short stout morphology that resulted from Xdsh-D2 expression in the dorsal vegetal blastomeres was surprising, as it is distinctly different from previously reported convergent extension phenotypes. To ensure that the novel phenotype was not unique to the Xdsh-D2 construct, we targeted injections of another mutant of Xdsh, Xdd1. In previous studies, Xdd1 has been shown to inhibit convergent extension of the dorsal mesoderm without affecting cell fates (Sokol, 1996). Xdd1 generated distinctly different phenotypes when targeted to the mesoderm versus the ectoderm at similar frequencies to Xdsh-D2. When injected into the dorsal animal blastomeres, Xdd1 caused severe dorsal flexure and a failure to straighten the A/P axis (Fig. 2I), while targeting of Xdd1 to the dorsal vegetal blastomeres produced short and stout, but straight, embryos (Fig. 2J). Likewise, dorsal animal injections

inhibited neural tube closure, while dorsal vegetal injections did not (data not shown).

Xdsh-D2 is fused to GFP, and we used GFP fluorescence as a lineage tracer of the injected mRNAs to confirm that our targeted injection did result in differential expression of the construct. When injected into the dorsal animal blastomeres at the 16-cell stage, embryos developed with open neural plates (not shown) and severe dorsal flexure (Fig. 3B), and GFP fluorescence was observed only in the dorsalmost tissues of the embryo (Fig. 3C), though the exact location was difficult to discern because of the distortion in embryo morphology. By contrast, embryos injected into the dorsal vegetal blastomeres at the 16-cell stage developed with shortened A/P axes, but no flexure (Fig. 3D), and GFP fluorescence was observed in both dorsal mesoderm and anterior endoderm, but was excluded from the neural tube (Fig. 3E).

Expression of Xdsh-D2 or Xdd1 inhibits convergent extension of the neural ectoderm in Keller sandwich explants

Analysis of convergent extension can be complicated by other morphogenetic movements occurring in whole embryos. A variety of explant techniques have been developed to examine convergent extension directly, and Keller sandwich explants are well suited for examination of neural convergent extension (Keller and Danilchik, 1988; Keller et al., 2000). In these
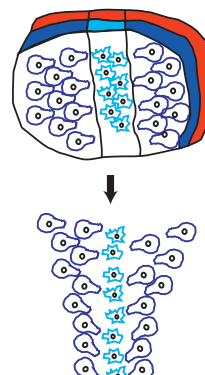

A. **Two domains of convergent extension**
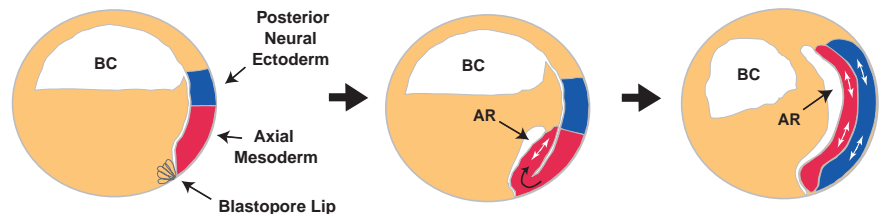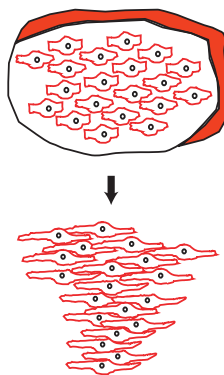
B. **Mesoderm**   **C.** **Neural over Mesoderm**   **D.** **Isolated Neural**
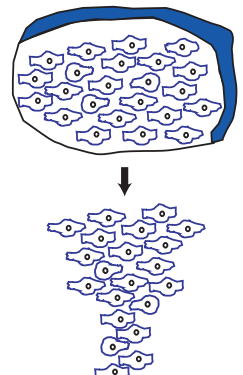
Fig. 1. (A) Convergent extension occurs in both dorsal mesodermal and posterior neural tissues. Axial mesoderm (red) involutes and undergoes convergent extension in parallel to convergent extension of overlying posterior neural ectoderm (blue). AR, archenteron; BC, blastocoel. (B-D) Cell behaviors during convergent extension. (B) Dorsal mesoderm (Shih and Keller, 1992a); (C) neural-over-mesoderm (Elul and Keller, 2000); (D) isolated posterior neural ectoderm (Elul et al., 1997). Red, mesoderm; Blue, posterior neural. In C, light blue indicates notoplate.

Fig. 2. Targeted injections of mutant Xdsh elicits distinctly different phenotypes. (A) Injection of Xdsh-D2 into the dorsal animal blastomeres at the eight-cell stage targets expression predominately to the neural ectoderm (blue); injection into the dorsal vegetal blastomeres targets expression to the dorsal axial mesoderm (red). (B) Control embryos at stage 20. (C) Dorsal-animal injection of Xdsh-D2 inhibits neural tube closure, and neural plates remain partially open at stage 20 (arrow). In other cases, neural plates close but fail to fuse normally (arrowhead). (D) Embryos injected dorsovegetally close their neural tubes normally; however, a few do display improper fusion of the neural folds (arrowhead). (E) Control embryos at stage 30. (F) At stage 30, embryos expressing Xdsh-D2 in the neural ectoderm exhibit a severe dorsal flexure and failure to straighten the A/P axis. (G) At stage 30, embryos expressing Xdsh-D2 in the mesoderm do not display dorsal flexure, but instead fail to elongate and are shorter and wider than controls. (H) Control embryo at stage 30. (I) Embryos injected with Xdd1 animally at the eight-cell stage. (J) Embryos injected vegetally at the eight-cell stage with Xdd1.
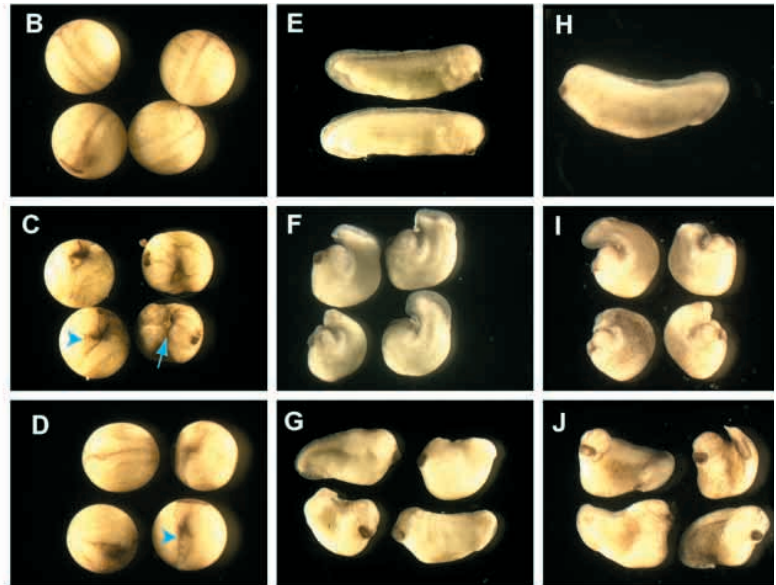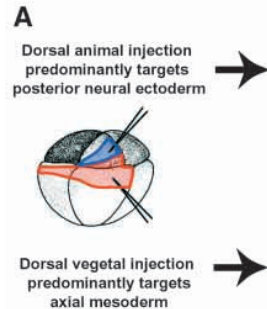
explants, two open-face dorsal marginal zone explants are cultured in tight apposition with their deep cells facing one another and superficial epithelia surrounding the entire explant (Fig. 4A); both the mesodermal and neural regions elongate and narrow significantly (Keller, 1991; Keller and Danilchik, 1988; Keller et al., 1992b). We therefore used Keller sandwiches to assess the effects of mutant Xdsh on neural convergent extension.

In control sandwich explants (Fig. 4B), both neural and mesodermal cells undergo convergent extension, and the two distinct domains of elongation can be observed. The mesoderm elongates less efficiently than does the neural tissue (Keller and Danilchik, 1988) and, as a result, does not narrow as extensively, forming a collar at the junction of the axial mesoderm and posterior neural ectoderm (Fig. 4A,B).

When Keller sandwiches were made from embryos injected with mRNA encoding Xdsh-D2, explants failed to elongate. In all cases, the neural ectoderm completely failed to elongate, and little elongation was observed in the mesoderm (Fig. 4C and see below). Furthermore, no distinction could be made between neural and mesodermal regions. Explants made from embryos expressing Xdd1 display the identical phenotype; they fail to elongate and no collar region can be identified (Fig. 4D).

It is important to note that targeted injection does not allow for expression exclusively in the ectoderm (Moody and Kline, 1990), and expression of Xdsh-D2 in the mesoderm likely accounts for its relatively weak elongation in experimental explants (Fig. 4C,D). Nonetheless, as we were interested in examining neural convergent extension, injections for this experiment were biased to the neural ectoderm, and GFP fluorescence indicated that Xdsh-D2 was present predominantly in the neural portion of explants (Fig. 4E). Mesodermal tissue lacking GFP fluorescence did elongate (Fig. 4E, arrows).
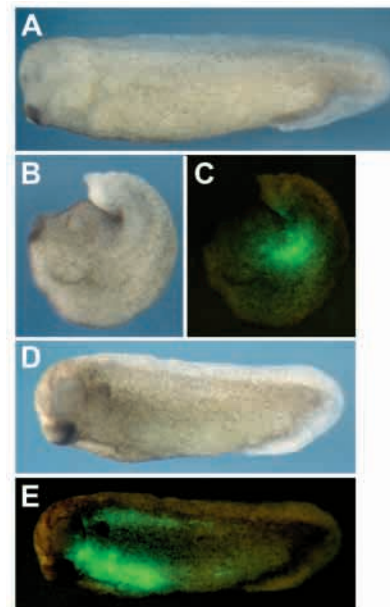
Fig. 3. Lineage tracing of targeted injections. (A) Control embryo. (B) Embryo injected with Xdsh-D2 into the dorsal animal two blastomeres at the 16-cell stage. Embryo displays severe dorsal flexure. (C) Fluorescence view of embryo in B; GFP fluorescence is present in the dorsalmost tissues and absent from ventral tissues. (D) Embryo injected with Xdsh-D2 into the dorsal vegetal two blastomeres at the 16-cell stage, showing short stout morphology. (E) Fluorescence view of embryo in D; GFP fluorescence is present in the both dorsal and ventral tissues and is excluded from the neural tube.

Inhibition of neural convergent extension by Xdsh-D2 is not secondary to changes in cell fate

The data above demonstrate that interference with non-

Fig. 4. Mutant Xdsh inhibits neural convergent extension in Keller sandwich explants. (A) Keller sandwiches were prepared by removing the entire dorsal marginal zone, which consists of dorsal axial mesoderm (AM, red), posterior neural ectoderm (PNE, blue), and some anterior ectoderm (yellow), from two different embryos. These two explants are cultured together with the deep cells facing, and superficial epithelia (black) surrounding the recombinate. Convergent extension of neural and mesodermal portions transforms the initially rectangular explant into a stereotyped morphology with two distinct domains of elongation and a collar region at the interface. (B) The stereotypical morphology of elongated Keller sandwiches in control explants (*n*=15). (C) Explants expressing Xdsh-D2 fail to elongate and no distinction can be made between mesodermal and ectodermal regions (*n*=11/11 display reduced elongation). (D) Explants expressing Xdd1 also fail to elongate (*n*=5/5 display reduced elongation). (E) Observation of GFP localization (green) demonstrates targeting of injected Xdsh-D2 to the neural ectoderm. Upper panels show explant morphology, lower panels show distribution of Xdsh-D2 GFP fluorescence (green). Note that the mesoderm does not express Xdsh-D2 (arrowheads) and has elongated; the adjacent neural tissue is expressing Xdsh-D2 (green) and has failed to elongate or narrow.
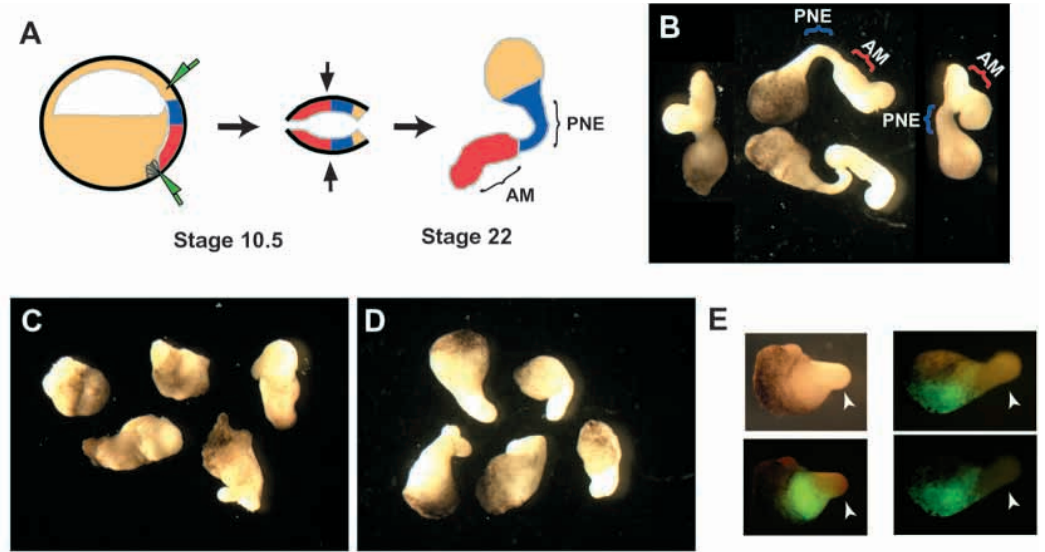
canonical Wnt signaling by expression of Xdsh-D2 or Xdd1 inhibits neural convergent extension. As only posterior neural cell types undergo convergent extension (Keller et al., 1992b), this phenotype could be a secondary consequence of anteriorization of cell fate in the neural tube. In order to ensure that posterior neural cell fates are not altered by Xdsh-D2, we examined the expression of the posterior neural marker *HoxB9* (Sharpe et al., 1987).

In control embryos, *HoxB9* is expressed throughout the spinal cord (Fig. 5A). Likewise, embryos injected into dorsal

Fig. 5. Xdsh-D2 expression does not inhibit posterior neural cell fates. (A) Control embryo stained for HoxB9. HoxB9 staining is normal, extending from behind the head (arrow) to the tip of the tail (arrowhead). (B) Embryos injected animally at the eight-cell stage with 1 ng of Xdsh-D2; HoxB9 staining is present from the back of the head to the tip of the tail. (C) In embryos in which the posterior neural folds have completely failed to meet, two separate domains of HoxB9 expression can be seen. (c′) Anterior view of the embryo in C shows the two domains of HoxB9 expression, one on each side, extending from behind the head (arrows) to the posterior limit of each side of the embryo (arrowheads). (D) Control embryo stained for Krox20. (E) Krox-20 staining pattern is normal in embryos injected animally with Xdsh-D2 mRNA at the eight-cell stage. (F) In some severely affected embryos, Krox20 staining pattern is mildly distorted, but both stripes are discernible. (d′-f′) Detail of staining patterns for embryos in D-F; arrowhead marks stripe in rhombomere 3; arrows mark rhombomere 5. (G-H) Xdsh-D2 expression in the mesoderm does not inhibit posterior neural cell fates. (G) Control embryos stained for HoxB9 and Krox20. (H) Embryos injected with 1ng of Xdsh-D2 mRNA into the dorsal vegetal blastomeres at the eight-cell stage and stained for HoxB9 and Krox20.
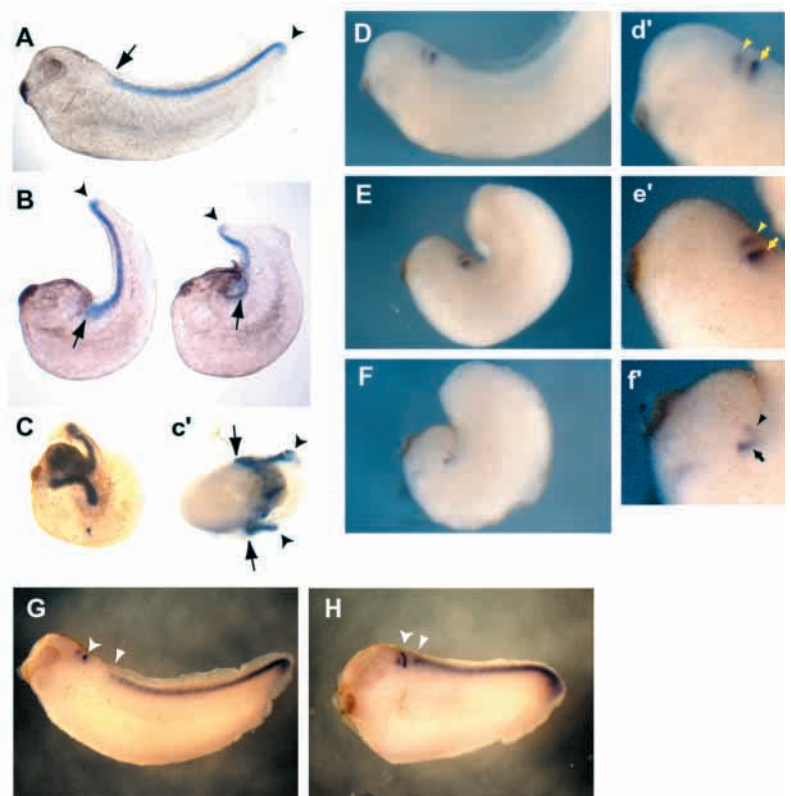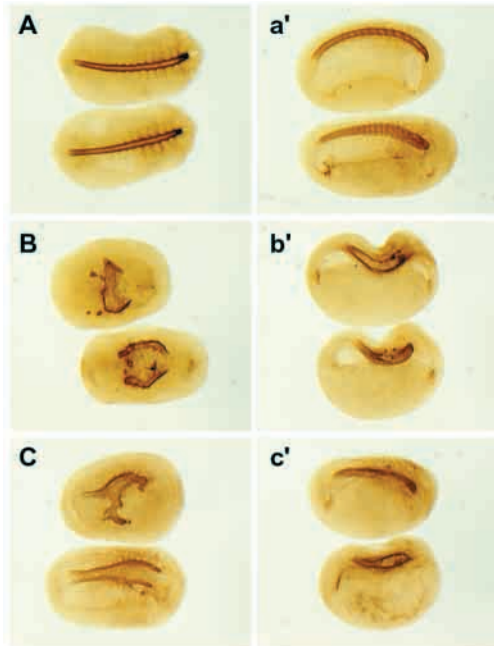
Fig. 6. Xdsh-D2 expression does not inhibit notochord differentiation. (A) Control embryos stained for notochord-specific antigen Tor70 (dorsal view, anterior towards the right). (B) Embryos injected dorsoanimally with Xdsh-D2. (C) Embryos injected dorsovegetally with Xdsh-D2. Side views of embryos are shown in a′-c′.

Fig. 7. Xdsh-D2 inhibits convergent extension in XBF-2-expressing animal caps. (A) Control uninjected animal caps do not elongate (*n*=0/40). (B) Animal caps injected with 0.5 ng of XBF-2 elongate as a result of neural convergent extension. Some caps elongate significantly (left); others elongate slightly (middle); and some caps fail to elongate (right). 45% of caps elongated to some degree; *n*=55. (C) Co-expression of 1 ng of Xdsh-D2 with 0.5 ng of XBF-2 strongly suppresses elongation in all caps. Very weak elongation (see caps at far left) was observed in only 7% of caps (*n*=45).

animal blastomeres with Xdsh-D2 mRNA (Fig. 5B) express *HoxB9* from just behind the head (arrows) all the way to the tip of the tail (arrowheads), despite the severe dorsal flexure.

As mentioned above, these embryos display defects in neural tube closure, and at later stages, these defects can be roughly categorized as partial or complete failure of neural tube closure. In embryos that display partial failure, the anterior and posterior regions of the neural tube close, while a large portion in the middle remains open. In these embryos, a single line of HoxB9 expression can be seen in the more posterior regions of the embryo, while in the central regions of the embryo where the neural tube has failed to close, HoxB9 is expressed in two distinct domains on either side of the open neural tube (not shown). In embryos that completely fail to close their neural tubes, the two domains of HoxB9 extend along each side of the embryo from the hindbrain level to the tailbud (Fig. 5C, c′). These data demonstrate that the most posterior cell fates of the neural tube are not inhibited by expression of Xdsh-D2, suggesting that Xdsh-D2 directly interferes with morphogenetic cell movements.

We also examined expression of the hindbrain marker *Krox-20* (Bradley et al., 1993). In control embryos, *Krox-20* is expressed in two discrete stripes in rhombomeres 3 and 5 (Fig. 5D,d′). Likewise, in embryos which display severe dorsal flexure after injection of Xdsh-D2 into the dorsal animal blastomeres, both bands can be distinguished (Fig. 5E,e′). In some of the more severely affected embryos, the orientation of the bands was sometimes distorted (Fig. 5F,f′), however no dramatic disruptions of *Krox-20* expression were observed.

Because targeted injections do not allow for exclusive expression of Xdsh-D2 in either mesoderm or ectoderm, it is
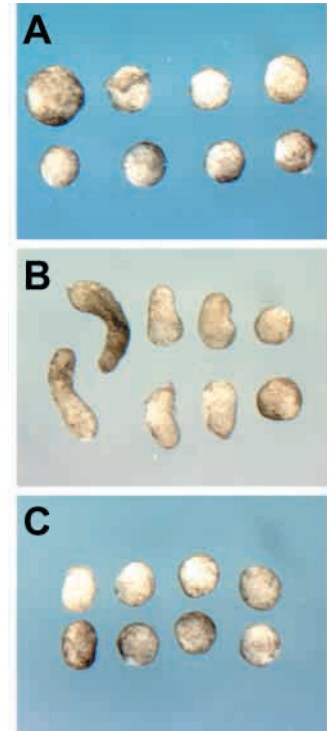
also important to rule out the possibility that expression of Xdsh-D2 in the mesoderm in some way alters neural cell fates and consequently inhibits neural convergent extension. However, both *HoxB9* and *Krox-20* staining is normal in embryos injected dorsovegetally with Xdsh-D2 (Fig. 5H). There is a slight decrease in the distance between the posterior Krox20 staining and the anteriormost *HoxB9* staining in Xdsh-D2 injected embryos, probably due to the failure of the embryo to elongate.

Finally, we examined cell fate specification in the dorsal mesoderm using the notochord specific antibody Tor70 (Bolce et al., 1992). Notochords of control embryos were elongate and sometimes mildly flexed toward the ventral side (Fig. 6A,a′). In both animally and vegetally injected embryos, notochords remained very broad, and some notochords were bifurcated (Fig. 6B,C). In no case did Xdsh-D2 expression eliminate the notochord, consistent with the finding that inhibition of Xdsh function in the mesoderm with Xdd1 also suppresses convergent extension without affecting cell fates (Sokol, 1996). In embryos injected dorsoanimally, notochords were dorsally flexed (Fig. 6b′), while in dorsovegetally injected embryos, notochords were severely shortened, but remained straight (Fig. 6c′).

The data above indicate that the failure of neural convergent extension in embryos injected with mutant *Xdsh* is not a secondary effect of changes in cell fate.

Xdsh-D2 inhibits convergent extension of XBF-2 expressing animal caps

Examination of the effects of mutants of *Xdsh* in both whole embryos and Keller sandwich explants strongly indicates that Xdsh is required for convergent extension in the neural ectoderm. Nonetheless, targeted injections do not allow for expression of mutant Xdsh constructs in the ectoderm

exclusively, and while neural convergent extension is autonomous, it can be modulated by signals from the mesoderm, both by vertical interactions (Elul and Keller, 2000; Elul et al., 1997) and by planar interactions (Keller et al., 1992c). As Xdsh-D2 strongly inhibits convergent extension of the mesoderm, it is important to rule out the possibility that any observed effect of Xdsh-D2 on neural convergent extension is not a secondary result of expression of mutant *Xdsh* in the adjacent mesoderm.

To address this issue, we first examined the effects of Xdsh-D2 on neural convergent extension in the absence of mesoderm using animal caps expressing the transcription factor *XBF-2*, which can neuralize animal caps without inducing mesoderm (Mariani and Harland, 1998). Uninjected animal caps did not elongate when removed at stage 9 and cultured until stage 20 (Fig. 7A). However, *XBF-2* can induce hindbrain and spinal cord cell fates (Mariani and Harland, 1998), and, as a result of neural convergent extension, nearly half of animal caps expressing *XBF-2* elongated to some degree (Fig. 7B). In contrast, elongation was strongly suppressed in caps co-injected with *XBF-2* and Xdsh-D2; only a very few caps elongated and that elongation was very subtle (Fig. 7C).

Xdsh-D2 inhibits convergent extension of neural plate grafts

Heterochronic grafting can be used to replace the neural plates of uninjected embryos with animal caps from injected embryos, and these grafted animal caps cells are induced to take on neural cell fates (Fig. 8A,B; Mariani et al., 2001; Ribisi et al., 2000). This approach allows expression of genes solely in the neural plate and not in the adjacent mesoderm. Importantly, these grafts will undergo normal neural morphogenesis, including convergent extension of the posterior neural plate (Fig. 8B,b'). We made grafts of animal caps expressing either GFP or Xdsh-D2 into uninjected embryos and assessed convergent extension by observing the fluorescent graft (Fig. 8B-E,b'-e'). When neural plates were replaced with GFP-expressing animal caps, the initially square graft converged and extended significantly (Fig. 8b'), and GFP-positive cells were observed throughout the entire A/P extent of the embryo at stage 30 (Fig. 8C,c'). However, when neural plates were replaced with Xdsh-D2 expressing animal caps, grafts failed to converge and extend, remained very short, and were not distributed throughout the A/P axis (Fig. 8D,d',E,e'). These results demonstrate that Xdsh-D2 expression solely in the neural ectoderm is sufficient to inhibit neural convergent extension, indicating a role for Xdsh signaling in this process.

Inhibition of neural convergent extension by grafting of Xdsh-D2-expressing cells allowed the effects of mutant *Xdsh* on neural morphogenesis to be assessed directly, without expression in the mesoderm. While grafts made with GFP-expressing cells formed normal neural tubes which closed and fused (Fig. 8C), grafts made with Xdsh-D2 failed to form closed neural tubes (Fig. 8D,E, arrows), consistent with the effects of targeted Xdsh-D2 expression (Fig. 2C).
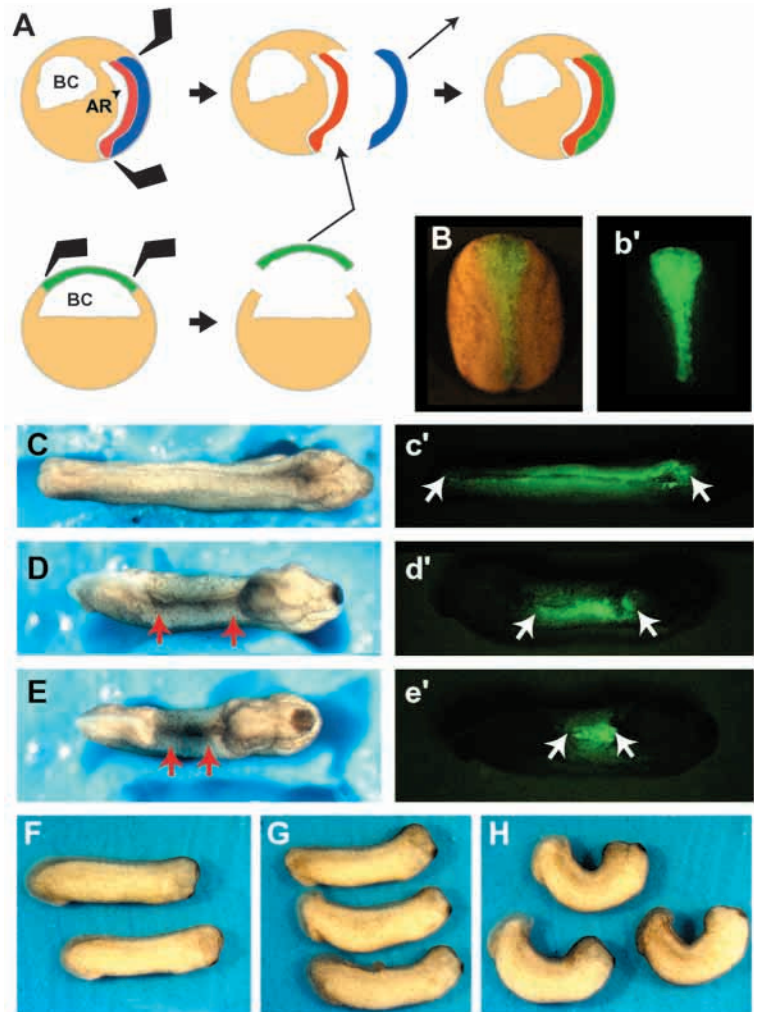

Fig. 8. Xdsh-D2 inhibits convergent extension in heterochronic neural plate grafts. (A) Neural plate grafts were prepared by removing the neural ectoderm of stage 11.5 embryos and grafting in animal caps from injected embryos. (B) Grafted GFP expressing animal caps are induced to become neural tissue (Mariani et al., 2001; Ribisi et al., 2000), are integrated into the host embryo, and undergo normal neural morphogenesis as shown by the convergent extension of the posterior portion of a GFP-expressing graft (b'). (C) Dorsal view of embryos with GFP-expressing grafts develop normally and close their neural tubes; GFP expression (c') demonstrates the extent of convergent extension of the graft (*n*=9 recombinates from three experiments). White arrows indicate the anterior and posterior limits of the GFP-expressing graft. (D,E) Dorsal view of embryos with Xdsh-D2-expressing grafts that fail to close their neural tubes (red arrowheads); GFP fluorescence of the Xdsh-D2 construct (d',e') indicates that the grafts fail to converge and extend (*n*=14 recombinates from three experiments). (F) Unmanipulated embryos at stage 30 are straight and elongated. (G) Embryos with GFP-expressing grafts are also elongated and straight, although they do display a very subtle dorsal flexure as a result of grafting. (H) Embryos with Xdsh-D2 grafts are elongated but develop with a pronounced dorsal flexure.

Neural convergent extension is necessary for complete elongation of the A/P axis

The grafting experiments also provided information about the forces that elongate the normal A/P body axis. Unmanipulated embryos elongate and straighten the body axis between gastrulation and tadpole stages (Fig. 8F). Likewise, embryos
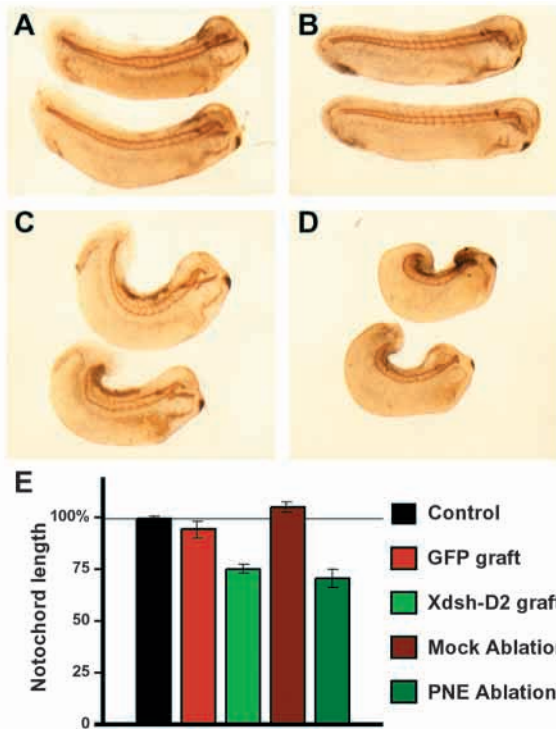
Fig. 9. Neural convergent extension is required for elongation of the A/P axis. (A-D) Tor70 notochord staining of manipulated embryos. GFP grafted (A) and mock-operated (B; see Materials and Methods) embryos develop with normal A/P axes. Embryos with Xdsh-D2-expressing grafts into the neural plate (C) or with ablated posterior neural plates (D) develop with severe dorsal flexure. (E) Quantitation of notochord length in manipulated embryos, expressed relative to length of unmanipulated control embryos. Control, *n*=12; GFP-grafted, *n*=6; Xdsh-D2 grafted, *n*=10; mock, *n*=4; ablated, *n*=4. Data shown are mean±s.e.m.

grafted with GFP-expressing cells also elongate and straighten (Figs 8G, 9A). However, embryos grafted with Xdsh-D2-expressing cells developed with a pronounced dorsal curvature (Figs 8H, 9C). Similar dorsal flexure was observed after manual ablation of the posterior neural plate (Fig. 9D), while mock-operated sibling embryos developed normally (Fig. 9B).

We next examined the contribution of neural convergent extension to axial elongation by measuring the notochords of grafted and ablated embryos. The notochords of GFP-grafted embryos and of mock-operated embryos were not changed compared with unmanipulated sibling control embryos (Fig. 9A,B,E). However, in Xdsh-D2 grafted embryos, there was a consistent reduction in notochord length (Fig. 9C,E). Embryos in which the posterior neural plate had been ablated and no graft put in its place displayed a similar degree of reduction in notochord length (Fig. 9D,E).

Other Wnt signaling components affect neural convergent extension

Several different components of the Wnt signaling network have been implicated in mesodermal convergent extension. In particular, *Xenopus Wnt5a* (*Xwnt5a*) and the interfering mutant of *Xenopus* Frizzled-8 (Nxfz-8) have both been shown to inhibit the non-canonical Wnt signals which control mesodermal convergent extension (Deardorff et al., 1998;
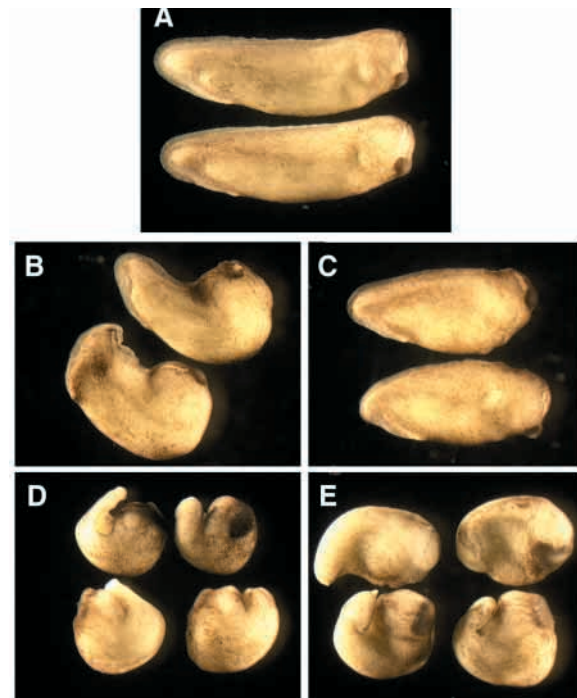

Fig. 10. Targeted injection of other Wnt antagonists has differential effects. (A) Control embryos. (B) Embryos injected animally with Xwnt5a mRNA display dorsal flexure. (C) Embryos injected vegetally with Xwnt5a are not 'kinked' but are foreshortened. (D) Embryos injected animally with Nxfz-8 mRNA display severe dorsal flexure. (E) Embryos injected vegetally with Nxfz-8 display two phenotypes. Many embryos do not display dorsal flexure (top), similar to other vegetally injected embryos in this study. Other embryos do display dorsal curvature (bottom). This phenotype may be due to higher diffusibility of the small, secreted Nxfz-8 protein.

Moon et al., 1993; Wallingford et al., 2001b). As Xdsh affects neural convergent extension, we also tested *Xwnt5a* and Nxfz-8 for their effects on neural convergent extension.

Xwnt5a has been shown to be an effective antagonist of mesodermal convergent extension, but does not inhibit dorsal cell fates (Moon et al., 1993; Torres et al., 1996). When *Xwnt5a* mRNA was injected into the dorsal animal blastomeres at the eight-cell stage, embryos developed with an obvious dorsal kink at about the level of the hindbrain (Fig. 10B). This flexure is less dramatic than that observed for Xdsh-D2 or Xdd1, but is consistent with the phenotype elicited by *Xwnt5a* injection at earlier stages (Moon et al., 1993). When *Xwnt5a* was targeted vegetally, no dorsal flexure was observed, and embryos developed with straight, but shortened, A/P axes (Fig. 10C). Likewise, when observed at stage 20, animally injected embryos displayed a failure of neural tube closure, while vegetally injected embryos displayed only defects in neural fold fusion (data not shown).

Nxfz-8, the extracellular domain of *Xfz-8*, has been shown to be a potent antagonist of *Xfz-8* activity, and also strongly and directly inhibits mesodermal convergent extension (Deardorff et al., 1998). As with other Wnt components examined in this study, Nxfz-8 arrested neural tube closure when injected into the dorsal animal blastomeres at the eight-cell stage (not shown), and these embryos developed with severe dorsal flexure at stage 30 (Fig. 10D). When injected into

Fig. 11. Parallel forces in axial elongation. (A,B) In normal embryos, elongation of the posterior neural ectoderm (blue), dorsal mesoderm (red), and ventral tissues (green) all contribute to the generation of a straightened and elongate A/P axis. (C) In embryos injected dorsoanimally with Xdsh-D2, strong inhibition of neural convergent extension and mild effects on mesodermal convergent extension conspire to generate a dorsally kinked and foreshortened A/P axis. (D) In dorsovegetally injected embryos, strong inhibition of mesodermal convergent extension results in a shortened axis, while neural and ventral elongation produce a straight axis. (E) In grafted Xdsh-D2 embryos, the embryo is dorsally curved, owing to the failure of neural convergent extension, and mesodermal convergent extension elongates the axis, though not completely.
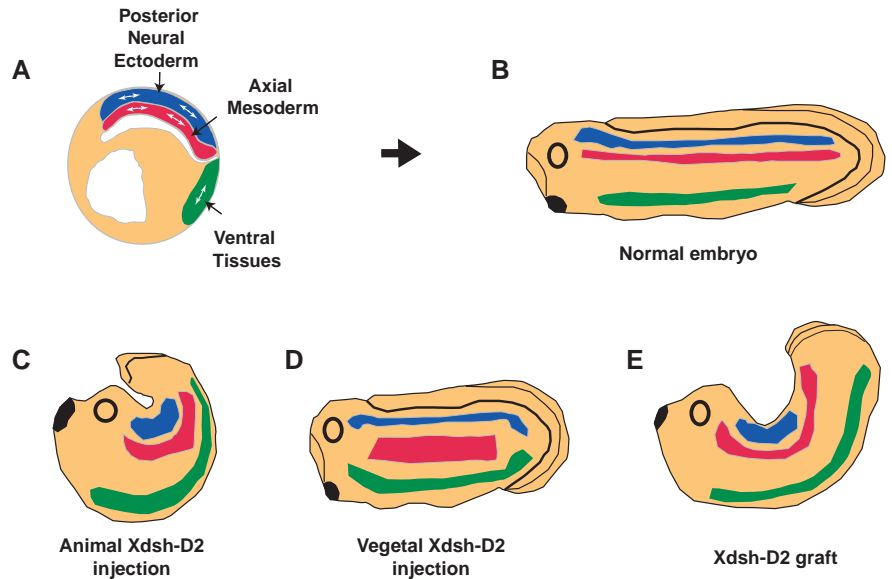
the dorsal vegetal blastomeres with Nxfz-8 mRNA, about half of the embryos developed with straight, short A/P axes (Fig. 10E, top embryos), while the other half developed with dorsal flexure more characteristic of animally injected embryos (Fig. 10E, bottom embryos). As Nxfz-8 consists of only the extracellular domain of *Xfz-8* and is secreted rather than membrane associated (Deardorff et al., 1998), its diffusibility may account for its effects on neural convergent extension when targeted to the mesoderm.

The differential effects of Nxfz-8 and *Xwnt5a* when targeted to ectoderm versus mesoderm support the idea that Wnt signals modulate convergent extension in both neural and mesodermal tissues.

DISCUSSION

A common mechanism regulating convergent extension in vertebrate tissues

Examination of the cell behaviors that drive convergent extension in mesodermal and neural tissues in *Xenopus* has revealed striking similarities, but also important differences (Fig. 1; Elul and Keller, 2000; Elul et al., 1997; Keller et al., 2000; Shih and Keller, 1992a). As such, it is unclear whether or not the same or similar molecular mechanisms control convergent extension cell movements in the different germ layers. The expression of *Xdsh* and *Frizzled* receptors in both dorsal mesoderm and posterior neural ectoderm (Deardorff et al., 1998; Djiane et al., 2000; Medina et al., 2000; Sokol et al., 1995) suggest that Wnt signals may be involved in controlling morphogenetic movements in both tissues. We have examined this possibility by testing the effects of Wnt pathway antagonists which inhibit mesodermal convergent extension on similar movements in the posterior neural ectoderm. Expression of several such Wnt signaling components had differential effects when targeted predominantly to mesoderm or ectoderm in whole embryos (Figs 2, 10), and these phenotypes are consistent with inhibition of mesodermal or neural convergent extension, respectively (see below).

Furthermore, Xdsh antagonists clearly inhibited autonomous neural convergent extension in Keller sandwich explants (Fig. 4), in animal caps (Fig. 7) and in neural plate grafts (Fig. 8). In situ hybridization and antibody staining of whole embryos indicate that these effects are not the result of changes in cell fates (Figs 5, 6).

Our data demonstrate that Xdsh signaling is a common mechanism that controls convergent extension in both dorsal mesoderm and posterior neural ectoderm of *Xenopus*. Despite the differences in cell fate and also in cell behaviors (Fig. 1), polarity decisions clearly play a key role in intercalation in both tissues. Our data support the hypothesis that a common molecular mechanism underlies the establishment of polarity in both neural and mesodermal convergent extension, and additional layers of regulation account for the differences in behaviors (Keller et al., 2000).

Finally, it should be noted that a similar mechanism of Wnt signaling controls convergent extension movements not only in *Xenopus* dorsal mesoderm and neural ectoderm, but also in the marginal zone of the zebrafish gastrula (Heisenberg et al., 2000). As such, the mechanisms that regulate convergent extension may be highly conserved throughout vertebrate tissues. Indeed, recent reports demonstrate that intercellular Ca^{2+} waves are also a common feature of convergent extension in all three of these tissues (Gilland et al., 1999; Leclerc et al., 2000; Wallingford et al., 2001a). It will be interesting to determine if similar mechanisms regulate convergent extension in invertebrate embryos, such as *Drosophila* (Irvine and Wieschaus, 1994) or sea urchins (Hardin, 1996).

Neural and mesodermal convergent extension cooperate with ventral elongation to form the anteroposterior body axis

During early *Xenopus* development, the elongation of the A/P axis of the trunk is driven by three autonomously elongating regions (Fig. 11A,B). The posterior neural ectoderm (hindbrain and spinal cord) and the underlying dorsal mesoderm (notochord and somites) elongate by convergent extension (Keller, 1975; Keller, 1976), and these movements are

autonomous in each germ layer (Keller and Danilchik, 1988) Rearrangements of ventral cells autonomously extend the ventral portion of the embryo (Drawbridge and Steinberg, 2000; Larkin and Danilchik, 1999). In light of these observations, the two phenotypes that result from targeted injection predominantly in the ectoderm versus the mesoderm are consistent with failure of convergent extension in each tissue individually.

When injection is targeted to the dorsal-animal blastomeres, neural convergent extension is strongly blocked, and elongation of the dorsal mesoderm will also be affected. The elongation of the ventral tissues is unaffected, and such uneven elongation then buckles the embryo, resulting in severe dorsal flexure (Fig. 11C). This buckling can be clearly observed in embryos stained for spinal cord (Fig. 5B) or notochord (Fig. 6B).

However, when injection is targeted to the dorsal vegetal blastomeres, convergent extension is strongly blocked in the mesoderm, while neural convergent extension is much less affected. Failure of mesodermal convergent extension restricts the elongation of the A/P axis, and elongation of the neural ectoderm is equalized by that of the ventral tissues yielding short, stout embryos (Fig. 11D). Indeed, a slight ventral buckling of the elongating neural tube can be observed in the posterior spinal cord of these embryos (Fig. 5H), though notochords remain straight (Fig. 6C).

The failure of neural and ventral elongation to extend the A/P axis when mesodermal convergent extension is suppressed suggests that mesodermal convergent extension may be the key force elongating the axis. Nonetheless, the consistent reduction in notochord length that results from inhibited neural convergent extension (Fig. 10E) demonstrates that neural elongation does contribute actively to the extension of the A/P axis. As such, Xdsh-D2 grafted embryos develop with only a slightly shorter notochord, and elongation of the axial mesoderm and ventral tissues around the immobile neural tissue produces the observed dorsal flexure (Fig. 11E).

Together, these results implicate mesodermal convergent extension as the primary force in A/P axis elongation and a more minor role for neural convergent extension in this process. However, neural convergent extension is clearly required in parallel with mesodermal convergent extension for the generation of a straightened body axis.

Importance of targeted mRNA injection in *Xenopus* embryos

The identification of two distinct convergent extension phenotypes that results from subtly different injection sites is important, especially because dorsal injection of Wnt components at the four-cell stage generally results in dorsal flexure, a phenotype produced primarily by the failure of neural, not mesodermal, convergent extension. Indeed, generation of these two very different phenotypes does not require injection at the eight-cell stage; simply biasing injections animally or vegetally at the four-cell stage can generate the two distinct phenotypes (data not shown). While many components may be common to convergent extension mechanisms in both dorsal mesoderm and neural ectoderm, not all molecular controls will necessarily be the same in both tissues. As such, it is important to assess effects on convergent extension accurately in each tissue. More generally, these

results highlight the importance of accurately targeting and tracing mRNA injections in *Xenopus* embryos.

The role of Xdsh and convergent extension in neural tube closure

Embryos that express Xdsh-D2 in the neural plate fail to close their neural tubes. In some cases, this failure may be a consequence of bifurcation of the notochord (Fig. 6B). However bifurcated notochords were not observed in embryos where Xdsh-D2 was expressed exclusively in the neural plate by grafting (not shown), though these embryos did fail to close their neural tubes (Fig. 8).

The open neural plate of these embryos is a striking phenotype, and it is particularly interesting that the failure of tube closure is restricted to the hindbrain and spinal cord. This phenotype may reflect a similarity to region-specific neural tube defects in humans, such as spina bifida or rachischisis, in which the cranial neural tube closes normally while more caudal regions fail to close (Juriloff and Harris, 2000). As such, it is possible that Xdsh and Wnt signaling plays a direct role in neural tube closure. Consistent with this hypothesis, analysis of cell behaviors during *Xenopus* neurulation has demonstrated that neural tube closure involves polarized protrusive activities in a number of cell types in the neural tube (Davidson and Keller, 1999). As Xdsh is directly involved in controlling both polarity and stability of similar protrusions in the mesoderm (Wallingford et al., 2000), these findings raise the possibility that Xdsh plays a similar role during neurulation.

While it is possible that Xdsh is directly involved in neural tube closure, it is also likely that the observed neural tube closure defects are to some degree a secondary consequence of a failure of convergent extension of the neural plate prior to overt neurulation. Several lines of evidence suggest that convergent extension of the neural plate contributes significantly to normal neural tube formation in amphibians (Davidson and Keller, 1999; Jacobson, 1994; Jacobson and Gordon, 1976) and also in the chick (Jacobson, 1984). Thus, the failure of neural tube closure in these embryos may be secondary to their failure to straighten the A/P axis, as curvature of the A/P axis has been shown to inhibit posterior neuropore closure in amniotes (Brook et al., 1991; van Straaten et al., 1993). The amphibian embryo may therefore provide a very simple model system in which to explore these neural tube defects.

Control of convergent extension cell movements by Xdsh is independent of the canonical Wnt pathway

In *Drosophila*, Dishevelled functions to transduce signals along at least two different Wnt pathways: the canonical Wnt/β-catenin pathway and the planar cell polarity pathway (Boutros and Mlodzik, 1999). Both of these pathways are also used in the *Xenopus* mesoderm. Canonical Wnt signals induce dorsal cell fates in the early embryo (Miller et al., 1999), and ectopic Wnt signaling on the ventral side induces a secondary axis (Sokol et al., 1991). During gastrulation, non-canonical Wnt signals similar to PCP signaling control convergent extension of the dorsal mesoderm (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000; Wallingford et al., 2001b). Our data strongly indicate that a similar molecular mechanism is at work during convergent extension of the posterior neural ectoderm.

The distinction between canonical and non-canonical pathways is illustrated by the similar effects of Xdsh-D2 and Xdd1 on neural convergent extension (Figs 2, 4). Both Xdsh-D2 and Xdd1 have been shown to severely inhibit convergent extension in the dorsal mesoderm (Wallingford et al., 2000). However, the two constructs have opposite effects in secondary axis assays, Xdsh-D2 is fully functional for canonical Wnt signaling (Rothbächer et al., 2000), while Xdd1 inhibits these signals (Sokol, 1996). As these two mutants of Xdsh have indistinguishable effects on convergent extension in both neural ectoderm (this study) and dorsal mesoderm (Wallingford et al., 2000), Xdsh-mediated control of convergent extension is likely to be independent of canonical Wnt pathways in both tissues.

This role for non-canonical Wnt signaling is particularly intriguing in the neural tube, where canonical Wnt/β-catenin signaling plays an important role in specifying cell fates (Baker et al., 1999; McGrew et al., 1997; McGrew et al., 1995). For example, expression of Xdsh-D2 inhibits convergent extension, but does not significantly alter the A/P pattern of the neural tube. However, inhibition of canonical Wnt signaling in the neural tube has dramatic effects on this pattern (McGrew et al., 1997; McGrew et al., 1995). As such, Xdsh may function simultaneously via canonical and non-canonical pathways to control both cell fate and cell motility in the posterior neural ectoderm.

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