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Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway

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SUMMARY

Gastrulation in the amphibian embryo is driven by cells of the mesoderm. One of the genes that confers mesodermal identity in *Xenopus* is *Brachyury* (*Xbra*), which is required for normal gastrulation movements and ultimately for posterior mesoderm and notochord differentiation in the development of all vertebrates. Xbra is a transcription activator, and interference with transcription activation leads to an inhibition of morphogenetic movements during gastrulation. To understand this process, we have screened for downstream target genes of Brachyury (Tada, M., Casey, E., Fairclough, L. and Smith, J. C. (1998) *Development* 125, 3997-4006). This approach has now allowed us to isolate *Xwnt11*, whose expression pattern is almost identical to that of *Xbra* at gastrula and early neurula stages. Activation of *Xwnt11* is induced in an immediate-early fashion by *Xbra* and its expression in vivo is abolished by a dominant-interfering form of *Xbra*, *Xbra-En^R*. Overexpression of a dominant-negative form of *Xwnt11*, like overexpression of *Xbra-En^R*, inhibits convergent extension movements. This inhibition can be rescued by *Dsh*, a component of the Wnt signalling pathway and also by a truncated form of *Dsh* which cannot signal through the canonical Wnt pathway involving GSK-3 and β-catenin. Together, our results suggest that the regulation of morphogenetic movements by Xwnt11 occurs through a pathway similar to that involved in planar polarity signalling in *Drosophila*.

Key words: *Xenopus*, *Brachyury*, Target gene, Wnt, Dishevelled, Gastrulation

INTRODUCTION

The mesoderm of the amphibian embryo arises through an inductive interaction in which blastomeres of the vegetal hemisphere of the embryo act on overlying equatorial cells (Nieuwkoop, 1969). Once mesoderm is induced, gastrulation takes place through coordinated morphological movements of the three germ layers. These movements, which include convergent extension and involution, are driven predominantly by cells of the mesoderm (Keller et al., 1992; Keller, 1986). This is particularly obvious during convergent extension, when polarised axial mesoderm cells intercalate in radial and mediolateral directions to cause dramatic elongation of the dorsal marginal zone (Shih and Keller, 1992; Wilson and Keller, 1991). The molecular mechanisms by which mesodermal cells become polarised and drive these movements are, however, poorly understood.

The mesoderm-inducing factor activin causes prospective ectodermal cells to undergo convergent extension (Symes and Smith, 1987) and also to activate the expression of mesoderm-specific genes in an immediate-early fashion. Of these genes, *Xenopus Brachyury* (*Xbra*), which is expressed in a widespread fashion throughout the mesoderm (Smith et al., 1991), is of some interest. *Brachyury* function is required for posterior mesoderm and notochord differentiation in mouse, zebrafish and *Xenopus* embryos (Conlon et al., 1996; Herrmann et al., 1990; Schulte-Merker et al., 1994), and mis-expression of *Xbra* in prospective ectodermal tissue of *Xenopus* causes ectopic mesoderm formation in a dose-dependent fashion (Cunliffe and Smith, 1992; O'Reilly et al., 1995). Of particular relevance to this study, we note that chimeric genetic studies in mice demonstrate that *Brachyury* is necessary for normal morphogenetic movements during gastrulation (Wilson et al., 1995). Furthermore, the morphogenetic movements of activin-treated animal caps are inhibited by a dominant-negative *Xbra* construct (Conlon and Smith, 1999). Together, these results indicate that *Brachyury* plays a key role not only in mesoderm formation but also in morphogenesis. Since Xbra functions as a transcription activator (Conlon et al., 1996; Kispert et al., 1995), it is likely that Xbra targets include genes that are required for convergent extension.

To investigate this issue, we have turned to a subtracted library in which Brachyury-inducible genes are highly enriched (Tada et al., 1998). This screen has allowed us to isolate *Xwnt11*, which was originally identified as a maternally expressed *Wnt* gene (Ku and Melton, 1993). Expression of *Xwnt11* in the marginal zone of the *Xenopus* embryo requires *Xbra* function, and induction of *Xwnt11* by hormone-inducible *Xbra* does not require protein synthesis (Saka et al., 2000), suggesting that it is activated by Xbra in a direct fashion.

To investigate the function of *Xwnt11* during gastrulation, we have constructed a C-terminally truncated form of the

protein (dn-wnt11) which, by analogy with a similar *Xwnt8* construct (Hoppler et al., 1996), should act in a dominant-negative fashion. Control experiments show that dn-wnt11 can specifically downregulate a hyper-phosphorylated form of Dishevelled (Dsh), a component of the Wnt signalling pathway, but is specific in the sense that it does not inhibit signalling by Xwnt8. Overexpression of *dn-wnt11* in intact *Xenopus* embryos and in dorsal marginal zone tissue impairs gastrulation movements without affecting mesodermal differentiation. Furthermore, like dominant-negative *Xbra*, *dn-wnt11* blocks activin-induced elongation of animal caps, suggesting that *Xbra* and *Xwnt11* act in the same genetic pathway to control gastrulation. Rescue experiments with downstream components of the Wnt signalling pathway and with different Dsh mutants demonstrate that *Xwnt11* does not mediate gastrulation through the canonical Wnt signalling pathway involving GSK-3 and β-catenin (reviewed by Cadigan and Nusse, 1997). Rather, it may act in a pathway similar to that involved in establishing planar polarity in *Drosophila* (Adler, 1992).

MATERIALS AND METHODS

Embryonic manipulations and cell adhesion assay

Fertilisation, culture and microinjection of *Xenopus* embryos were as described (Tada et al., 1997). Embryos were staged according to Nieuwkoop and Faber (1975). A unit of activin activity is defined by Cooke et al. (1987). The cell adhesion assay was carried out as described (Smith et al., 1990).

Isolation of *Xwnt11* from a subtracted library

Screening of a cDNA library enriched for Xbra target genes was as described (Tada et al., 1998). Sequencing analysis of PCR fragment pPCR121 revealed it to be *Xwnt11* (Ku and Melton, 1993). Induction of *Xwnt11* by *Xbra-GR* was confirmed by northern blotting as described below.

Construction of a dominant-negative Wnt11 and deletion mutants of Dsh

A C-terminally truncated dominant-negative *Xwnt11* construct (comprising amino acids 1-282) was generated according to Hoppler et al. (1996). PCR was carried out using pGEM-X9 (Ku and Melton, 1993) as a template and the primers 5′-CCCCCTCGAGAGTACCA-ATGGCTCCGACCCG-3′ and 5′-TTGGAGATCTTTCAGCAGTA-GTCAGGGGAAC-3′. The PCR product obtained was cloned into the *Xho*I and *Bgl*II sites of pSP64TXB. The coding region of full-length *Xwnt11* was also amplified by PCR and cloned into pSP64TXB to generate pSP64T-Xwnt11.

Various truncated forms of Dsh were generated by PCR using Xdsh-myc (Sokol, 1996) as a template. PCR fragments were then cloned into pCS2-myc. Constructs were as follows: Dsh-ΔN (amino acids 178-736), Dsh-ΔPDZ (amino acids 1-301 and 381-736, identical to Xdd1 of Sokol, 1996), Dsh-ΔC (amino acids 1-374: note that this differs from Xdd2 of Sokol, 1996), and Dsh-DEP+ (amino acids 337-736). Amino acid positions are according to Sokol et al. (1995).

All pSP64T constructs were linearised with *Bam*HI and transcribed with SP6 RNA polymerase. All pCS2-dsh constructs were linearised with *Not*I and transcribed with SP6 RNA polymerase. RNA was synthesized as described (Smith, 1993).

Western blotting, immunoprecipitation and phosphatase treatments

Animal caps or whole embryos previously injected with 200 pg *myc-dsh* RNA were extracted in a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail (Boehringer) in the presence of phosphatase inhibitors including 50 mM sodium fluoride, 5 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 10 μM okadaic acid. Protein extracted from the equivalent of two animal caps was analysed on a 7.5 % acrylamide gel by SDS-PAGE and then blotted to a PVDF membrane (Sequi-Blot, BIO-RAD) by wet electrophoretic transfer. The membrane was reacted with anti-myc monoclonal antibody 9E10 and subsequently with anti-mouse IgG conjugated with alkaline phosphatase (Sigma) followed by detection with NBT and BICP.

Extracts of five embryos previously injected with 200 pg *myc-dsh* RNA were processed for immunoprecipitation using anti-myc monoclonal antibody 9E10 essentially as described (Cunliffe and Smith, 1994). The immunoprecipitates were divided into three, washed with PAP buffer (40 mM MOPS, pH 5.5, 50 mM NaCl, 2 mM PMSF and protein inhibitor cocktail (Boehringer)) and treated with 0.12 units of potato acid phosphatase (Sigma) in the presence or absence of phosphatase inhibitors at 37°C for 1 hour. Samples were subjected to SDS-PAGE followed by western blotting with the anti-myc antibody.

In situ hybridization and immunocytochemistry

The protocol of Harland (1991) was used with minor modifications, including the use of BM purple as substrate and without RNAase treatment. An *Xwnt11* probe was prepared by linearising pGEM-X9 with *Xba*I and transcribing with SP6 RNA polymerase. The *Xbra* probe was as described (Tada et al., 1997). *Myf-5* and *goosecoid* probes were synthesized from the full-length cDNA clones (Blumberg et al., 1991; Hopwood et al., 1991). Whole-mount immunocytochemistry with monoclonal antibodies MZ15 (Smith and Watt, 1985) and 12/101 (Kintner and Brockes, 1984), specific for notochord and muscle respectively, was performed as described (Smith, 1993). Immunostaining was carried out essentially as described (Cunliffe and Smith, 1994).

RNA preparation, northern blotting and RNAase protection

RNA preparation and RNAase protection analyses were performed as described (Tada et al., 1997) except that lithium precipitation was omitted. Samples were analysed for expression of *Xbra* (Smith et al., 1991), *goosecoid* (Blumberg et al., 1991), *Xwnt8* (Christian et al., 1991), *Siamois* (Lemaire et al., 1995), actin (Mohun et al., 1984), *chordin* (Sasai et al., 1994), *Bix1* (Tada et al., 1998), *Xvent1* (Gawantka et al., 1995), *MyoD* (Hopwood et al., 1989) and ODC (Isaacs et al., 1992). To make an *Xwnt11* probe, pPCR121 was linearised with *Hin*fI and transcribed with T3 RNA polymerase. Northern blotting was performed as described (Sambrook et al., 1989).

RESULTS

Xwnt11 is a downstream target of Xbra

In an effort to isolate Xbra targets, we have constructed a cDNA library enriched for genes activated by a hormone-inducible *Xbra* construct (Tada et al., 1998). In a preliminary screen, we encountered two cDNAs out of 53 randomly picked clones that encode Xwnt11 (Ku and Melton, 1993). The ability of this gene to be induced by *Xbra-GR* was confirmed by northern blotting analysis using RNA derived from dexamethasone (DEX)-treated or untreated animal caps (Fig. 1A). A single transcript was strongly induced by *Xbra-GR* in the presence of DEX. Expression of *Xwnt11* is also induced by wild-type *Xbra* in animal caps (Casey et al., 1999).

The temporal and spatial expression of *Xwnt11* was studied

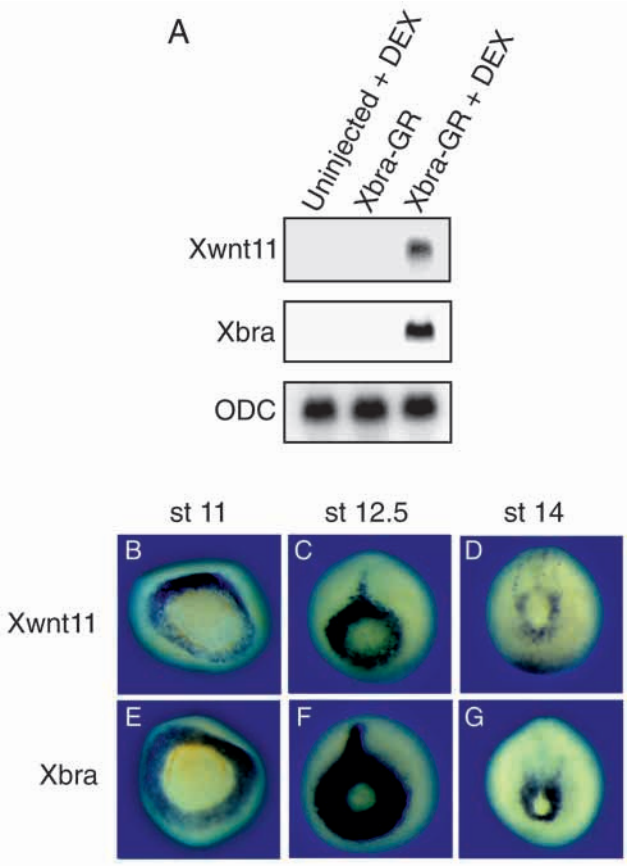

Fig. 1. Identification of *Xwnt11* as a target of Xbra. (A) Induction of *Xwnt11* by Xbra-GR. Animal caps derived from embryos injected with 50 pg *Xbra-GR* RNA or left uninjected were dissected at blastula stages and then treated with 10^{-6} M dexamethasone (DEX) for 3 hours or left untreated. RNA was extracted and analysed by northern blotting. *ODC* and *Xbra* served as loading and positive controls, respectively. Note that DEX alone does not induce expression of *Xwnt11*. (B-G) Comparison of expression patterns of *Xwnt11* (B-D) and *Xbra* (E-G) at stage 11 (B,E), stage 12.5 (C,F) or stage 14 (D,G).

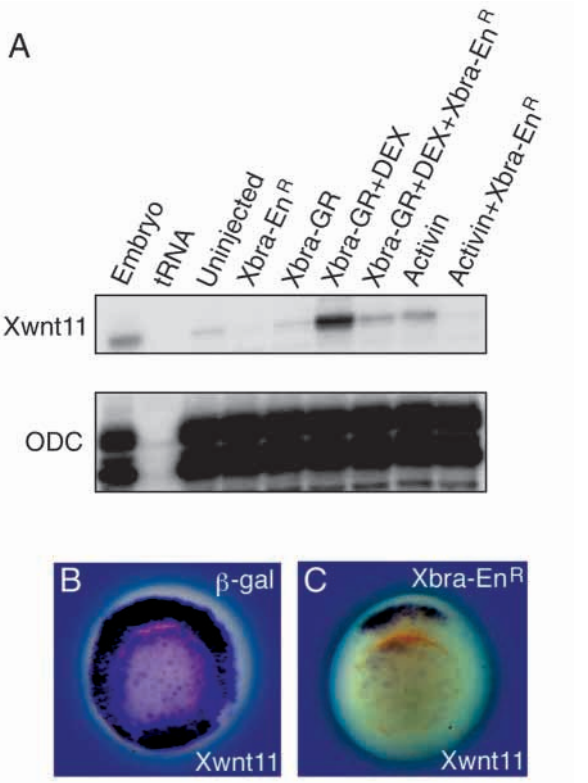

Fig. 2. Inhibition of *Xwnt11* expression by a dominant-interfering Xbra construct. (A) Expression of *Xwnt11* is induced in animal pole regions by activin (8 units/ml) or *Xbra-GR* in the presence of DEX, and induction is inhibited by *Xbra-En^R* (500 pg RNA). (B,C) Downregulation of *Xwnt11* by *Xbra-En^R* in the whole embryo. Embryos were injected with 500 pg *β-gal* RNA (B) or 500 pg *Xbra-En^R* RNA (C) into both blastomeres at the 2-cell stage. Expression of *Xwnt11* was examined by in situ hybridisation at the early gastrula stage. Note that injection of *Xbra-En^R* RNA causes a dramatic reduction in *Xwnt11* expression, although expression in dorsal superficial cells remains.

by in situ hybridisation and compared with that of *Xbra* (Fig. 1B-G). Zygotic expression of *Xwnt11*, like that of *Xbra*, occurs throughout the marginal zone of the embryo at the early gastrula stage and persists in posterior circumblastoporal tissue throughout gastrula stages to the early neurula stage. Detailed analysis using an Xbra-specific antibody also reveals coexpression of Xbra with *Xwnt11* (not shown). The expression pattern of *Xwnt11* is thus almost identical to that of *Xbra*, consistent with the suggestion that expression of *Xwnt11* at gastrula stages is regulated by *Xbra*. To test this idea, induction of *Xwnt11* by *Xbra* was examined both in animal caps and in whole embryos. In the animal cap assay, *Xwnt11* is induced by *Xbra-GR* in the presence of DEX as well as by activin. In both cases, induction is inhibited by a dominant-negative version of *Xbra*, *Xbra-En^R* (Fig. 2A). Furthermore, induction of *Xwnt11* by *Xbra-GR* is 'immediate-early' in the sense that it can occur in the presence of cycloheximide (Saka et al., 2000). When *Xbra-En^R* is expressed throughout the embryo (Fig. 2C), *Xwnt11* expression is abolished at the early gastrula stage, except in a small region in the dorsal side of the embryo, perhaps reflecting *Xwnt11* expression in the epithelial layer of the organiser where *Xbra* is not expressed (Glinka et al., 1996). Finally, we note that mis-expression of *Xwnt11* in animal caps does not induce ectopic *Xbra* expression (Glinka et al., 1996), and that expression of dominant-negative *Wnt11* does not inhibit expression of *Xbra* (see below). Together, these results suggest that expression of *Xwnt11* in posterior mesoderm is regulated by *Xbra*.

Construction of a dominant-interfering Wnt11

Previous overexpression studies have shown that *Xwnt11* has weak dorsalising activity (Glinka et al., 1996; Ku and Melton, 1993) and, when overexpressed in animal pole explants, it inhibits activin-induced elongation (Du et al., 1995). To test the requirement for *Xwnt11* function in the embryo, we employed a C-terminally truncated form of *Xwnt11* (*dn-wnt11*; Fig. 3A) which, by analogy with a similar truncated form of *Xwnt8*, should act in a dominant-negative fashion (Hoppler et al., 1996). We first tested the specificity of *dn-wnt11* by asking if it inhibits the dorsalising effects of *Xwnt8*. Complete secondary axes were induced by *Xwnt8* in 67% of cases (n=43). This frequency was reduced by truncated *Xwnt8* (7%, n=45), but not

by truncated *Xwnt11* (47%, *n*=53) (Fig. 3B-D). To confirm this observation, we examined expression of *Siamois*, a direct target of the canonical Wnt pathway (Brannon et al., 1997). Induction of *Siamois* by *Xwnt8* in ventral marginal zone tissue was blocked by *dn-wnt8*, but not by *dn-wnt11* (Fig. 3E), indicating that truncated *Xwnt11* does not interfere with signalling by Xwnt8.

The above observations do not address whether *dn-wnt11* does block the function of the wild-type protein. To investigate this question, we studied phosphorylation of Dsh (Yanagawa et al., 1995), an intracellular component of the Wnt signalling pathway (Sokol et al., 1995). When myc-tagged Dsh (*myc-dsh*) was expressed in embryos or in animal cap explants, two forms of the protein were detected by immunoblotting with anti-myc antibody (Fig. 4A). These two bands exist in similar proportions from early blastula to gastrula stages throughout the embryo (not shown). Although overexpression of *Xwnt11* or treatment with activin did not alter the relative abundance of the two bands in animal pole explants, expression of *dn-wnt11* caused the disappearance of the upper band (Fig. 4A). To investigate the possibility that the upper band corresponds to a phosphorylated form of Dsh, as seen in *Drosophila* (Yanagawa et al., 1995), immunoprecipitated myc-Dsh proteins were treated with potato acid phosphatase (PAP) in the presence or absence of phosphatase inhibitors. PAP treatment caused the mobility of both myc-Dsh bands to shift to the same size as that of in vitro translated myc-Dsh, and this shift was blocked by phosphatase inhibitors (Fig. 4B). These results suggest that the upper myc-Dsh band is a hyper-phosphorylated form that can be downregulated by *dn-wnt11*. Downregulation of this hyper-phosphorylated form of Dsh can be rescued by *Xwnt11* and the related *Xwnt5A* (Fig. 4C), confirming that members of this class of Wnt regulate hyper-phosphorylation of Dsh. These experiments, and those described in Fig. 3, indicate that truncated *Xwnt11* inhibits the function of the Wnt5A/Wnt11 class of Wnts, but not the function of the Wnt8 class. The biological significance of the hyper-phosphorylated form of Dsh is, at present, unknown, and is discussed below.

The ability of wild-type *Wnt11* to restore levels of hyper-phosphorylated Dsh (Fig. 4C) was corroborated by experiments in which the inhibition of activin-induced elongation caused by *dn-wnt11* (Fig. 5A,B; see below) was also rescued by wild-type *Wnt11*. Optimal rescue occurred with intermediate doses of *Xwnt11* (Fig. 5C), with high doses having little effect (Fig. 5D). Since wild-type *Xwnt11* also inhibits activin-induced elongation of animal caps (Du et al., 1995), this observation suggests that convergent extension requires precisely controlled levels of Wnt11 signalling.

A dominant-interfering Wnt11, like Xbra-En^{R}, inhibits formation of posterior structures

To examine the requirement for Xwnt11 function during development, we overexpressed *dn-wnt11* RNA throughout the embryo. Injection of *dn-wnt11* RNA caused a dramatic loss of posterior tissues while anterior structures appeared normal, except for slightly enlarged cement glands (Fig. 6A,E,F). This phenotype was presaged by incomplete gastrulation movements (Fig. 6D) and is quite similar to that caused by overexpression of dominant-negative *Xbra*, and a dominant-negative *dsh*, *Xdd1* (Sokol, 1996). Whole-mount immunocytochemistry using the notochord-specific antibody MZ15 and the muscle-specific antibody 12/101 showed that differentiation of notochord and muscle was unaffected by *dn-wnt11*, although the length of both tissues was reduced (Fig. 6E,F). To analyse these phenotypes in more detail, we examined mesodermal markers at the early gastrula stage. The pan-mesodermal marker *Xbra* and the dorsal and dorsolateral mesodermal markers *goosecoid* and *Myf-5* were unaffected by *dn-wnt11* (Fig. 6J-L). This observation differs from that observed following overexpression of *dn-wnt8*, when *MyoD* expression and muscle differentiation are reduced (Hoppler et al., 1996). Our results therefore suggest that interfering with *Xwnt11* function inhibits posterior extension of the embryo without affecting mesodermal differentiation.

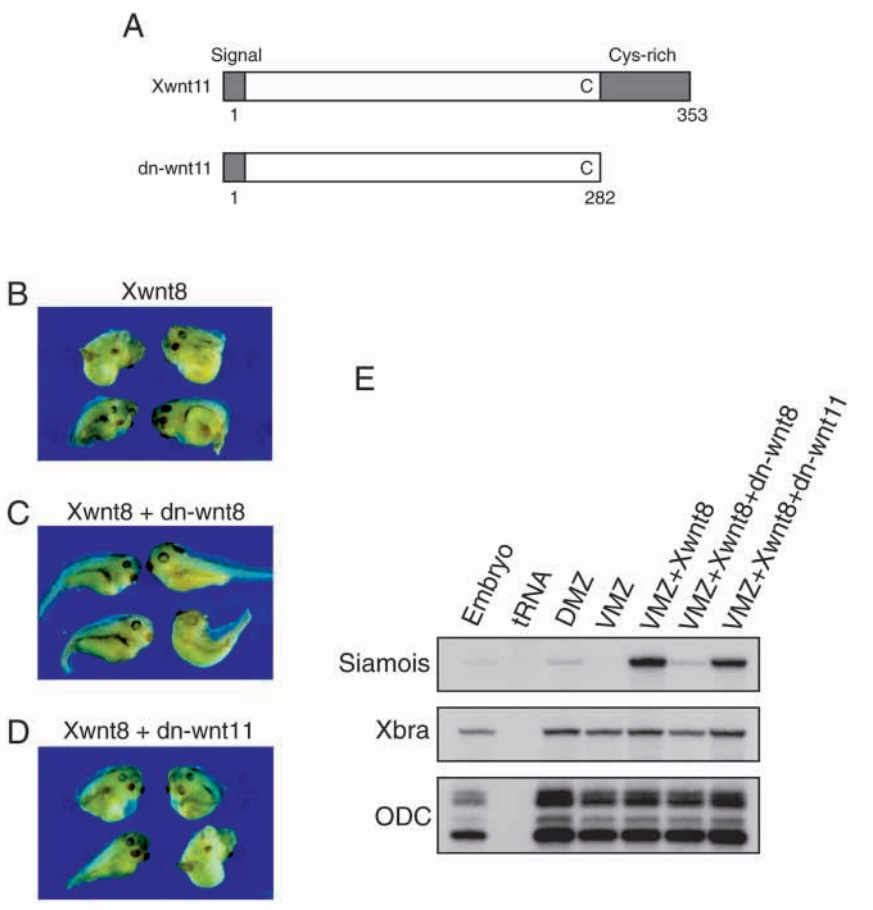

Fig. 3. Specificity of a dominant-negative *Wnt11*. (A) Schematic illustration of a C-terminally truncated Wnt11. (B-D) *Xenopus* embryos were injected with 1.2 pg *Xwnt8* RNA into one ventrovegetal blastomere at the 8-cell stage either alone (B) or in the presence of a 20-fold excess of *dn-wnt8* RNA (C) or *dn-wnt11* RNA (D). (E) RNAase protection analysis of ventral marginal zones derived from embryos at stage 10.5 following injection at the 2-cell stage with *Xwnt8* RNA along with *dn-wnt8* RNA or *dn-wnt11* RNA.

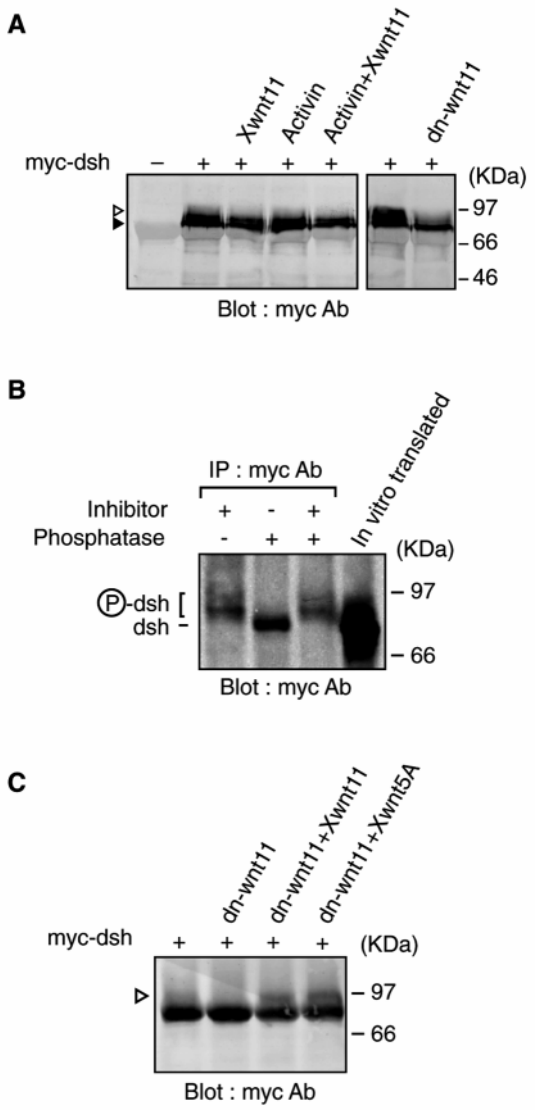

Fig. 4. Dn-wnt11 modulates phosphorylation of Dsh. (A) Dn-wnt11 downregulates a slow-migrating form of Dsh. (Left panel) Animal caps derived from mid-blastula (stage 8) embryos previously injected with 200 pg *myc-dsh* RNA either alone or with 250 pg *Xwnt11* RNA were treated or left untreated with activin (8 U/ml) and cultured to stage 10. (Right panel) Animal caps injected with 200 pg *myc-dsh* RNA either alone or with 2 ng *dn-wnt11* RNA were dissected at stage 10. Extracts from these caps were subjected to SDS-PAGE and western blotting and probed with an anti-myc antibody. Dn-wnt11 causes the downregulation of a slower-migrating form of myc-Dsh (open arrowhead) while another band remains essentially unchanged (closed arrowhead). (B) Exogenous Dsh is phosphorylated constitutively. Embryos injected with 200 pg *myc-dsh* RNA at stage 10 were extracted and subjected to immunoprecipitation using an anti-myc antibody. Immunoprecipitated Dsh proteins were left untreated in the presence of phosphatase inhibitors or treated with PAP in the presence or absence of phosphatase inhibitors. They were then subjected to SDS-PAGE and western blotting and detected using an anti-myc antibody. In vitro translated myc-Dsh protein is used to demonstrate the mobility of unmodified myc-Dsh. (C) A hyper-phosphorylated form of Dsh is regulated by *Xwnt11* or *Xwnt5A*. Animal caps from embryos injected with 200 pg *myc-Dsh* RNA together with 2 ng *dn-wnt11* RNA, 250 pg *Xwnt11* RNA or 250 pg *Xwnt5A* RNA were subjected to immunoblotting using an anti-myc antibody. Note that a hyper-phosphorylated form of Dsh is downregulated by dn-wnt11 and rescued by overexpression of *Xwnt11* and *Xwnt5A* RNA.

To analyse more directly the disruption of gastrulation caused by *dn-wnt11* (Fig. 6D), we studied dorsal marginal zone (DMZ) tissue. Control DMZ expressing β-gal underwent convergent extension movements (Fig. 7A), while elongation was inhibited by *dn-wnt11* (Fig. 7B). This inhibition occurred without affecting notochord differentiation (Fig. 7D) and without affecting expression of the dorsal mesodermal markers *goosecoid*, *chordin* and *MyoD*, the ventral mesodermal markers *Xwnt8* and *Xvent1*, or the panmesodermal markers *Xbra* and *Bix1* (Fig. 7E). The phenotypes caused by *dn-wnt11* are thus distinct from those of *dn-wnt8*, which causes a decrease in muscle differentiation (Hoppler et al., 1996). Overall, our results indicate that Wnt11 function is required for normal gastrulation movements, and particularly convergent extension, but not for mesodermal differentiation.

Xwnt11 and Dsh are required for convergent extension

Activin-induced elongation of animal pole explants provides a powerful model for the analysis of convergent extension (Deardorff et al., 1998; Kim et al., 1998; Sokol, 1996; Symes and Smith, 1987; Zhong et al., 1999). In previous work, we have shown that such elongation is inhibited by dominant-interfering *Xbra* (Conlon and Smith, 1999; Fig. 8C; Table 1), and we find that the same is true of dominant-negative *Xwnt11* (Figs 5B, 8D; Table 1). Although it inhibited elongation, *dn-wnt11* had no effect on the ability of activin to induce expression of *Xbra*, *goosecoid*, *Xwnt8* or *Bix1*, whereas *Xbra-En*^{R} blocks induction of *Xbra* and *Xwnt8* and, to a lesser extent, of *Bix1* (Fig. 8G; Conlon and Smith, 1999; Tada et al., 1998). These results are consistent with the suggestion that *Xwnt11* function is necessary for morphogenetic movements, but not for specification of mesoderm.

The phenotypes caused by *dn-wnt11* resemble those caused by a dominant-negative *Xdsh*, *Xdd1* (Fig. 8E; Sokol, 1996). This raises the possibility that interfering with the canonical Wnt signalling pathway might lead to an inhibition of elongation in the animal cap assay. To investigate this, we examined the effects of a dominant-negative Tcf-3 construct, ΔN-Tcf3, which is able to block the transcriptional function of β-catenin (Molenaar et al., 1996). Surprisingly, overexpression of *ΔN-Tcf3* failed to inhibit the elongation of animal caps in response to activin (Fig. 8F; Table 1), while only half the amount of RNA was sufficient to block *Xwnt8*-induced *Siamois* expression in ventral marginal zone tissue (not shown). These observations raise the possibility that the Wnt signalling pathway involved in the regulation of morphogenetic movements branches at Dsh.

Mapping of domains on Dsh involved in morphogenetic movements

The above results suggest that *Xwnt11* regulates morphogenetic movements in a manner that is independent of the canonical Wnt signalling pathway involving β-catenin and which does not affect cell adhesion. We therefore investigated the possibility that Xwnt11 acts in a pathway analogous to that involved in planar polarity signalling in *Drosophila*, in which the *dsh*^{1} allele causes defects in the orientation of ommatidia

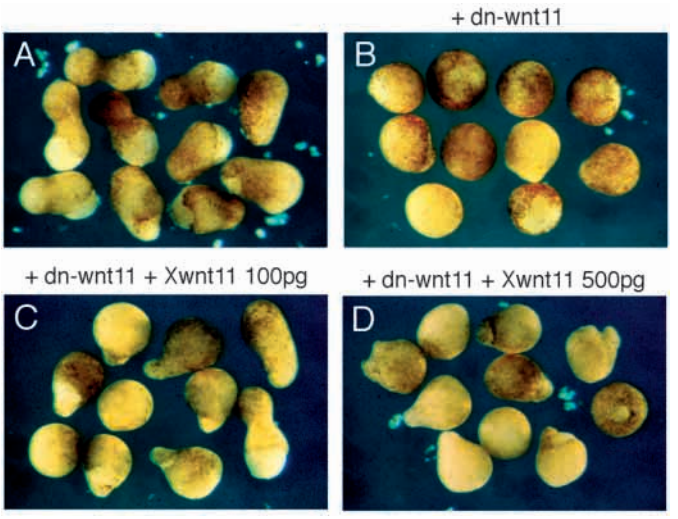

Fig. 5. Rescue of dn-wnt11 phenotype in animal cap explants in response to activin. (A) Animal caps derived from uninjected embryos treated with 8 units/ml activin and cultured to the equivalent of stage 18. (B-D) Animal caps derived from embryos injected with 2 ng *dn-wnt11* RNA (B) along with 100 pg (C) or 500 pg (D) *Xwnt11* RNA were treated with activin. Note that low (100 pg) doses of Xwnt11 partially rescue the inhibition of elongation caused by dn-wnt11 (19%, *n*=47), whereas high doses (500 pg) do so much less efficiently (4%, *n*=47).

in the eye and bristles of the wing with no other apparent phenotype (Adler, 1992).

It is possible to distinguish between the canonical Wnt pathway and the planar polarity pathway by use of constructs in which one or more of three domains of Dsh (DIX, PDZ and DEP) are deleted (Fig. 9A; see Discussion and Boutros and Mlodzik, 1999). Our data show that *Dsh-ΔPDZ* (which is identical to *Xdd1* of Sokol, 1996), blocks morphogenetic movements (Fig. 9E) but also inhibits the canonical Wnt pathway, as judged by its ability to prevent *Xwnt8*-induced activation of *Siamois* in ventral marginal zone tissue (Fig. 9I; see Brannon et al., 1997). *Dsh-ΔC* behaves in a similar fashion, although it inhibits elongation less effectively than *Dsh-ΔPDZ* (Fig. 9F,I; Table 1). In contrast, *Dsh-ΔN* has no effect in either assay (Fig. 9D,I; Table 1), while, significantly, *Dsh-DEP+* inhibits activin-induced elongation without inhibiting the canonical Wnt pathway (Fig. 9G,I; Table 1). Additional experiments showed that none of the deletion mutants, unlike wild-type Dsh, is able to induce expression of *Siamois* in ventral marginal zone tissue, indicating that they cannot alone activate the canonical Wnt signalling pathway (Fig. 9H).

Together, these results, and especially those obtained with *Dsh-DEP+*, confirm that the signalling pathway associated with elongation movements is distinct from the canonical Wnt pathway. They also reveal similarities between convergent extension movements in *Xenopus* and planar polarity signalling in *Drosophila* (see Discussion).

A Dsh construct that cannot signal via the canonical Wnt signalling pathway can rescue inhibition of activin-induced elongation by dn-wnt11

A Dsh construct comprising the PDZ and DEP domains without the DIX domain (Dsh-ΔN) does not inhibit activin-induced elongation of animal pole regions and nor does it activate, or block, the canonical Wnt signalling pathway (Fig. 9D,H,I). To investigate whether the PDZ and DEP domains of Dsh are sufficient to mediate morphogenetic movements, we asked whether Dsh-ΔN can rescue the inhibitory effects of *dn-wnt11* on activin-induced elongation. In doing so, we first characterised the effects of wild-type Dsh and Dsh-ΔN in more

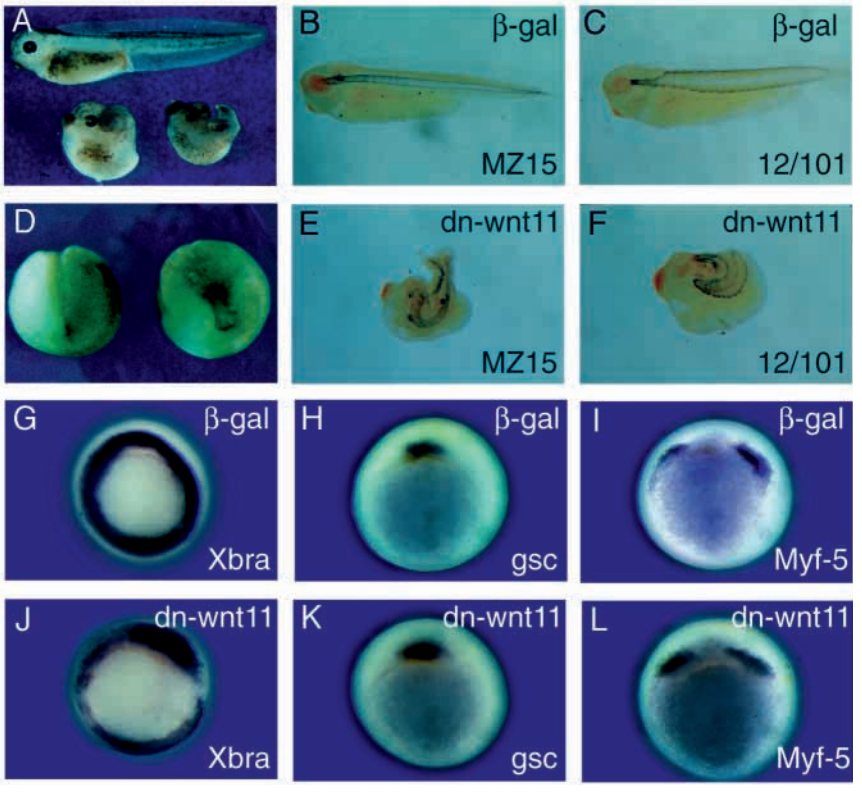

Fig. 6. Effects of *dn-wnt11* on whole embryos. (A) Control embryos injected with 2 ng *β-gal* RNA (upper) or embryos injected with 2 ng *dn-wnt11* RNA (lower) cultured to stage 34. Note that *dn-wnt11* inhibits formation of posterior structures (76%, *n*=78). (D) Control β-gal-expressing embryo (left) or dn-wnt11-expressing embryo (right) cultured to stage 14. Note that *dn-wnt11* inhibits gastrulation movements. (B,C) Control embryos injected with 2 ng *β-gal* RNA were stained at stage 34 with the notochord-specific monoclonal antibody MZ15 (B) or the muscle-specific monoclonal antibody 12/101 (C). (E,F) Embryos injected with *dn-wnt11* RNA stained with MZ15 (E), or 12/101 (F). *Dn-wnt11* does not affect notochord differentiation and although it reduces the size of the somites it does not affect muscle differentiation. (G,H,I) Embryos injected with 2 ng *β-gal* RNA into both blastomeres at the 2-cell stage stained for *Xbra* (G), *goosecoid* (H) or *Myf-5* (I) at stage 10.5. (J,K,L) Embryos at stage 10.5 previously injected with 2 ng *dn-wnt11* RNA and stained for *Xbra* (J), *goosecoid* (K) or *Myf-5* (L). Note that *dn-wnt11* affects none of these mesodermal markers.

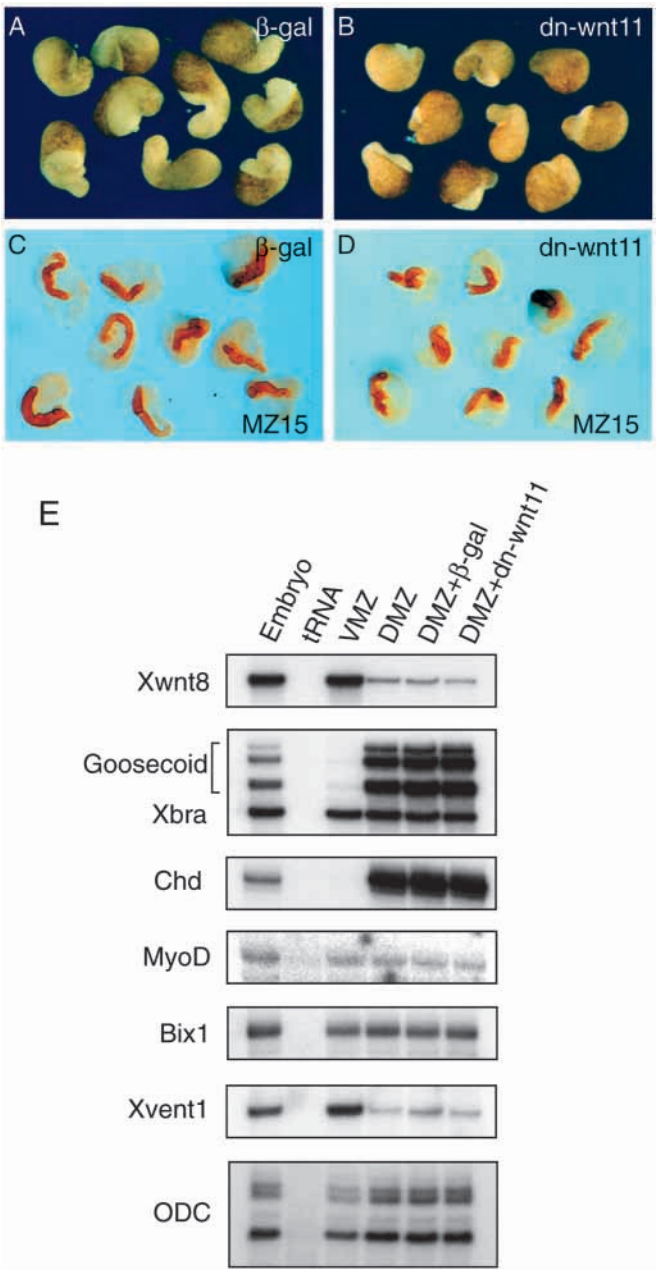

Fig. 7. Effects of *dn-wnt11* on dorsal marginal zone explants. (A-D) Dorsal marginal zones derived from embryos injected with 2 ng *β-gal* RNA (A,C) or 2 ng *dn-wnt11* RNA (B,D) into 2 dorsal blastomeres at the 4-cell stage were cultured to stage 18 (A,B) and at stage 34 they were stained with the notochord-specific monoclonal antibody MZ15 (C,D). *Dn-wnt11* inhibits elongation of DMZ explants, but does not affect notochord differentiation. (E) RNAase protection analysis detecting expression of mesodermal markers in DMZ explants derived from embryos injected with *β-gal* or *dn-wnt11* RNA. Markers were analysed at stage 10.5.

detail. Wild-type *Dsh*, unlike *Xwnt8*, has both mesoderm-inducing and dorsalising activities and causes elongation of animal caps without activin treatment (Itoh and Sokol, 1997; Fig. 10A). In contrast, *Dsh-ΔN* cannot induce mesoderm or cause dorsalisation (Fig. 10A), and it cannot make animal pole regions undergo convergent extension movements (data not shown). However, overexpression of *Dsh-ΔN* and, to a lesser extent, of wild-type *Dsh* was able to restore activin-induced elongation to animal caps expressing *dn-wnt11* (Fig. 10D,E; Table 1). This rescue is not due to induction of *Xwnt11* by *Dsh-ΔN* (not shown). We also note that *Dsh-ΔN* failed to restore elongation blocked by *Xbra-En^R* (not shown).

Table 1. Inhibition of activin-induced elongation in animal caps

Treatment	Elongation (%)			
	–	+	++	Number
Control	100	–	–	75
dn-wnt11	100	–	–	24
Dsh-ΔN	100	–	–	20
Activin	–	11	89	75
Activin + Xbra-En^R	98	2	–	48
Activin + dn-wnt11	89	11	–	59
Activin + dn-dsh	95	5	–	63
Activin + ΔN-Tef3	–	3	97	39
Activin + Dsh-ΔN	–	3	97	35
Activin + Dsh-ΔC	49	23	28	39
Activin + Dsh-DEP+	53	27	20	51
Activin + dn-wnt11 + Dsh	15	56	29	39
Activin + dn-wnt11 + Dsh-ΔN	13	36	51	39

–, no elongation; +, partial elongation; ++, strong elongation. dn-dsh is is identical to Dsh-ΔPDZ and Xdd1 (Sokol, 1996).

These results confirm that *Xwnt11* regulates convergent extension through a pathway that does not involve β-catenin, because *Dsh-ΔN* cannot activate this pathway (Fig. 9H). The fact that *Dsh-ΔN* cannot rescue the effects of *Xbra-En^R* suggests that other genes activated by *Xbra* are also involved in the regulation of convergent extension.

DISCUSSION

Xwnt11 is a target of Xbra

Previous studies indicate that *Brachyury*, an immediate-early target of mesoderm inducers, is required for gastrulation movements in *Xenopus* (Conlon and Smith, 1999) and in mice (Wilson et al., 1995). This requirement is particularly well illustrated in the animal cap assay, where activin induces expression of *Xbra* (Smith et al., 1991) and causes explants to extend and undergo convergent extension movements resembling those occurring during gastrulation (Symes and Smith, 1987): inhibition of Xbra function prevents such elongation (Conlon and Smith, 1999). Brachyury functions as a transcription activator (Conlon et al., 1996), suggesting that it regulates the expression of genes required for normal gastrulation movements. To identify such genes, we screened a subtracted library enriched for Xbra-inducible cDNAs (Tada et al., 1998) and, in this way, isolated *Xwnt11* (Figs 1, 2). Expression of *Xwnt11* is inhibited in embryos in which Xbra function is blocked (Fig. 2C), and induction of *Xwnt11* in response to hormone-inducible Xbra does not require protein synthesis (Saka et al., 2000), suggesting that *Xwnt11* is a direct target of Xbra. Use of a dominant-interfering *Xwnt11* construct (see below) does not inhibit expression of *Xbra* (Figs 6G, 7E, 8G), indicating that unlike *Xbra* and *eFGF* (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), *Xbra* and *Xwnt11* are not components of an indirect autoregulatory loop.

We note that, although the expression patterns of zebrafish *Wnt11* (*zwnt11*) and *Brachyury* (*ntl*) are virtually identical at shield stages, expression of *zwnt11* is little affected in *ntl* mutant embryos during gastrulation, although it is downregulated later (Makita et al., 1998). It is possible that expression of *zwnt11* during gastrulation is regulated by an additional copy of zebrafish *Brachyury*, which may have arisen during a genome duplication event (Amores et al., 1998). Alternatively, another T-box gene such as *spt* may also regulate *zwnt11* (Griffin et al., 1998); we have recently shown that the *Xenopus* homologue of *spt*, *VegT*, induces expression of *Xwnt11* in isolated animal pole regions (Casey et al., 1999). It will be interesting to examine *zwnt11* expression in *ntl*;*spt* double mutants.

Inhibition of Wnt11 function

To investigate the function of *Xwnt11* during gastrulation, we have made use of a C-terminally truncated form of the protein which, by analogy with a similar construct based on *Xwnt8* (Hoppler et al., 1996), would be expected to act in a dominant-negative fashion. Control experiments revealed that *dn-wnt11*, unlike *dn-wnt8*, did not interfere with the ability of wild-type *Xwnt8* to cause axis duplication in *Xenopus* embryos; nor did it block induction by *Xwnt8* of *Siamois* in ventral marginal zone tissue (Fig. 3). We also note that the effects of overexpression of *dn-wnt8* in intact *Xenopus* embryos differ from those obtained with *dn-wnt11*. In particular, *dn-wnt8* causes a reduction in muscle differentiation (Hoppler et al., 1996) which is not observed with *dn-wnt11* (Figs 6F,L, 7E).

When expressed in isolated animal pole regions, *dn-wnt11* downregulated a hyper-phosphorylated form of myc-tagged Dishevelled (Fig. 4A). The significance of this form of Dsh is, at present, unclear. One possibility is that it arises through the action of Wnt5A, which is expressed throughout the animal hemisphere of the gastrula embryo (Morgan et al., 1999). Consistent with this, we showed that the effect of *dn-wnt11* in downregulating hyper-phosphorylated Dsh can be rescued by wild-type *Wnt11* and also by *Wnt5A* (Fig. 4C). It is also possible that increased levels of Dsh (resulting from injection of RNA encoding myc-Dsh) sensitise animal caps to low levels of Wnt signalling. Consistent with this suggestion, overexpression of Dsh in *Drosophila* Schneider cells causes increased levels of a hyper-phosphorylated form of the protein (Yanagawa et al., 1995). In the future, it will be important to study the function of hyper-phosphorylated Dsh, to identify which amino acids are phosphorylated, and to investigate how phosphorylation is regulated.

These results are consistent with the idea that Wnt5A and Wnt11 are members of a family of Wnts that differs from a group including Wnt1 and Wnt8 (Du et al., 1995). They also show that the effects of *dn-wnt11* are specific in the sense that it inhibits Wnt11 signalling but not Wnt8. It remains possible, however, that *dn-wnt11* also inhibits the function of Wnt5A and other members of this class. Of the known members of the *Wnt5* family, however, only *Xwnt11* is expressed in the mesoderm of *Xenopus* during gastrulation (Du et al., 1995; Moon et al., 1993), suggesting that the effects of *dn-wnt11* on morphogenetic movements discussed below are due to inhibition of this family member. This conclusion is supported by genetic analysis in zebrafish showing that impaired

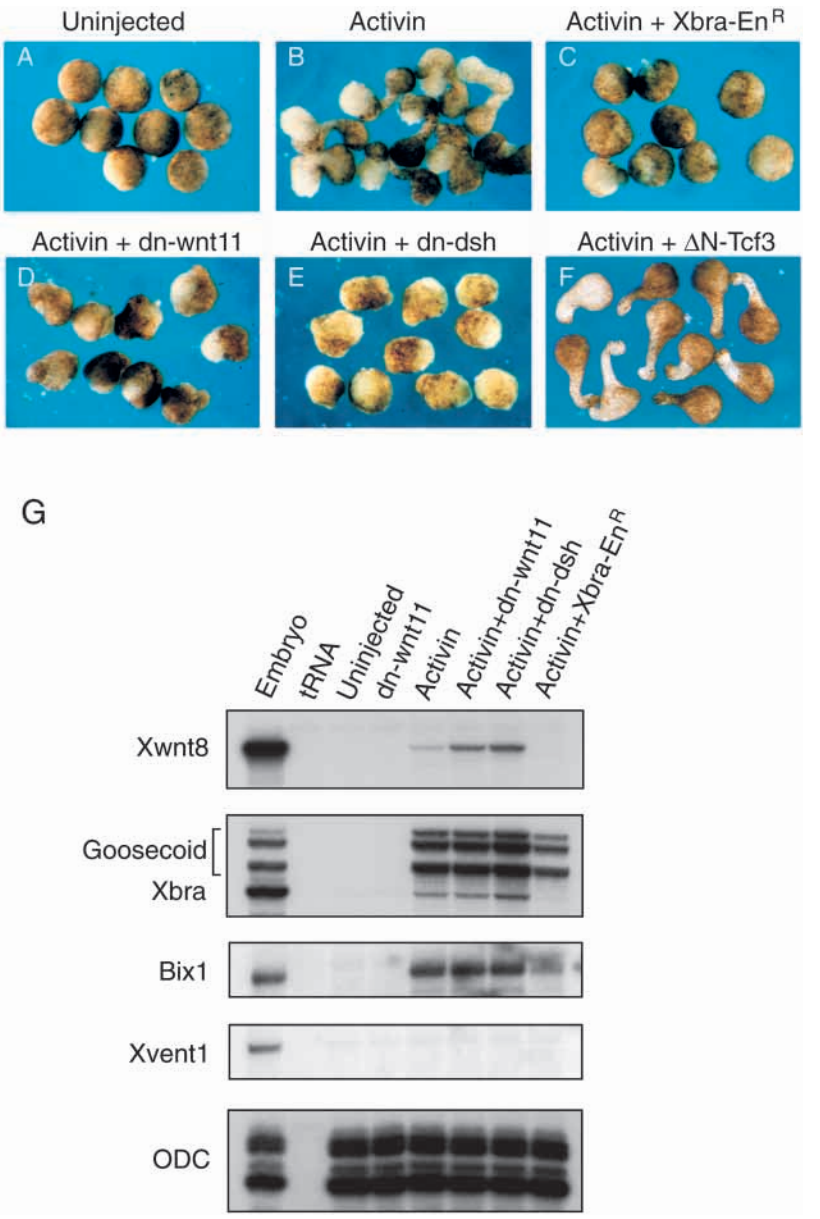

Fig. 8. Like *Xbra-En^R*, *dn-wnt11* inhibits elongation of animal cap explants in response to activin. Animal caps derived from uninjected embryos were left untreated (A) or treated with 8 units/ml activin (B) and cultured to the equivalent of stage 18. (C-F) Animal caps derived from embryos injected with 500 pg *Xbra-En^R* RNA (C), 2 ng *dn-wnt11* RNA (D), 2 ng *dn-dsh* (*Xdd1*) RNA (E), or 500 pg *ΔN-Tcf3* RNA (F) were treated with activin. Note that Xbra-En^R, dn-wnt11 and dn-dsh block elongation in response to activin, whereas ΔN-Tcf3 does not. (G) RNAase protection analysis detecting expression of mesodermal markers in animal caps derived from embryos injected with the above dominant-negative constructs. Markers were analysed at stage 10.5.

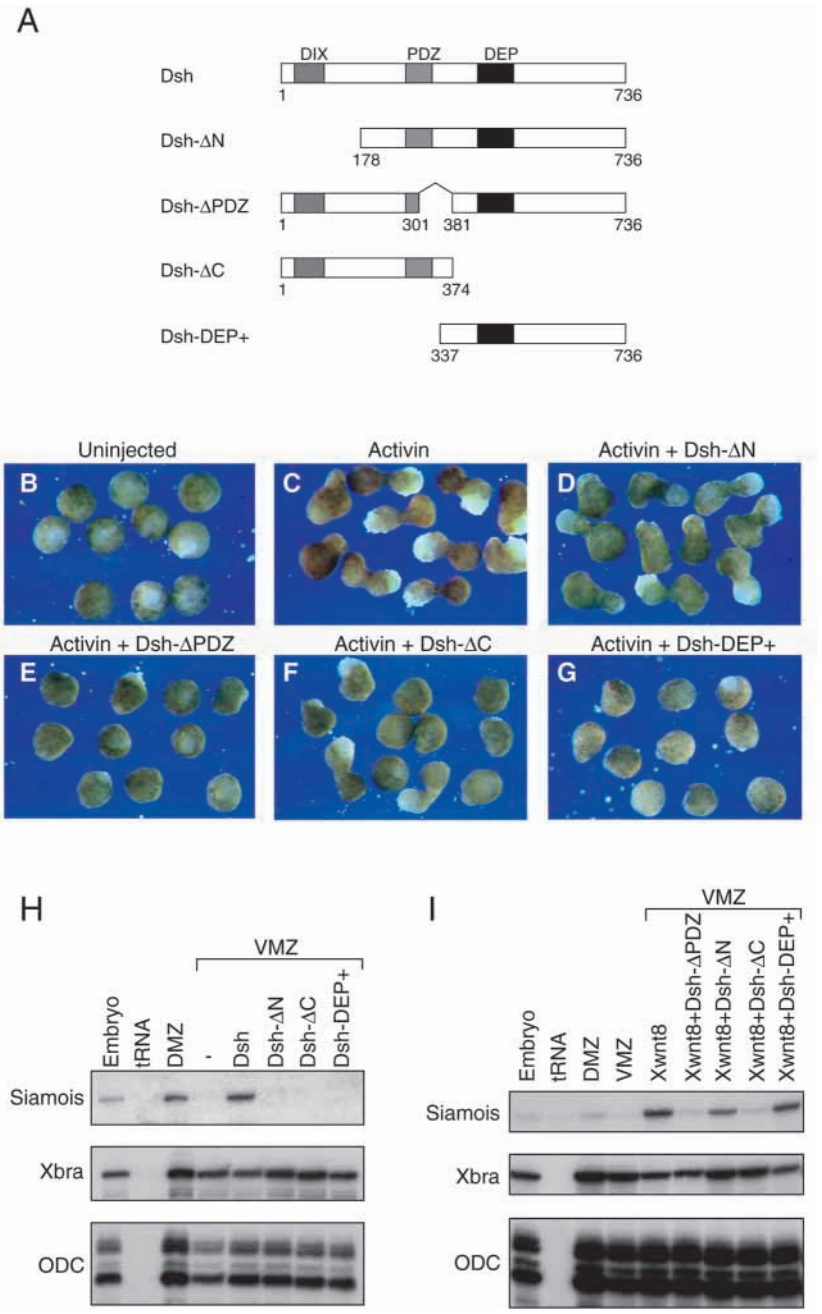

Fig. 9. Mapping of Dsh domains required for convergent extension movements. (A) Dsh deletion constructs. See Materials and Methods for details. (B-G) Animal pole explants derived from embryos injected at the 1-cell stage with RNA encoding different Dsh constructs were treated with activin (8 units/ml) at stage 8.5 and cultured until control embryos reached stage 18. (H) RNAase protection analysis illustrating the effects of Dsh deletion mutants on *Siamois*, a direct target of the canonical Wnt signaling pathway, in ventral marginal zone explants of the stage 10 embryo. (I) RNAase protection analysis illustrating the ability of different Dsh constructs to interfere with the ability of *Xwnt8* to induce expression of *Siamois* in ventral marginal zone explants.

convergent extension movements in *silberblick*$^{-/-}$ embryos, which carry a null mutation in the *Wnt11* gene, can be rescued by overexpression of *Wnt11* RNA (Heisenberg et al., 2000).

Xwnt11 is required for gastrulation movements

Use of *dn-wnt11* indicates that Xwnt11 activity, like that of Xbra, is required for gastrulation movements in whole embryos (Fig. 6D) and for convergent extension movements of dorsal marginal zone explants (Fig. 7) and activin-treated animal caps (Fig. 8). Unlike *Xbra*, however, *Xwnt11* is not required for mesodermal differentiation; its activities are restricted to the control of gastrulation. Although Xwnt11 activity is required for gastrulation movements in *Xenopus*, it is not sufficient. Overexpression of *Xwnt11* alone does not cause elongation of animal caps, and *Xwnt11* is not capable of rescuing activin-induced elongation in animal caps derived from embryos injected with RNA encoding Xbra-En^{R} (Table 1 and data not shown). This suggests that there are additional Xbra targets involved in the regulation of gastrulation.

What is the role of Xwnt11 during gastrulation? One possibility is that it regulates cell adhesion; Brieher and Gumbiner (1994) have shown that activin-induced elongation of animal pole regions is associated with a decrease in cadherin-mediated cell-cell adhesion. In principle, it is possible that such a decrease in adhesion might occur through Xwnt11-induced translocation of β-catenin from membrane to nucleus, but such a translocation has never been demonstrated and, as we discuss below, Xwnt11 seems not to exert its effects via β-catenin. Moreover, our experiments reveal that overexpression of wild-type *Xwnt11* or *dn-wnt11* has no effect on cell adhesion to fibronectin (data not shown). It seems more likely that changes in cell adhesion associated with gastrulation are regulated by expression of molecules such as paraxial protocadherin (PAPC) (Kim et al., 1998), which is required for activin-induced morphogenetic movements and whose expression is regulated by VegT/Xombi/antipodean/BraT, which itself is induced by activin in isolated animal pole regions (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996).

Another possibility is that Xwnt11 influences cell polarity. This idea is supported by evidence suggesting that Xwnt11 does not act through the canonical Wnt signalling pathway and by a comparison between convergent extension in *Xenopus* and planar polarity signalling in *Drosophila* (see below).

Wnt11 does not act through the canonical Wnt pathway to regulate gastrulation

Several lines of evidence suggest that Xwnt11 does not act to regulate gastrulation through the canonical Wnt signalling pathway involving GSK-3 and β-catenin. First, previous work has shown that unlike *Xwnt8*, which does act via β-catenin (Fagotto et al., 1997), *Xwnt11* does not induce anterior structures in *Xenopus* (Ku and

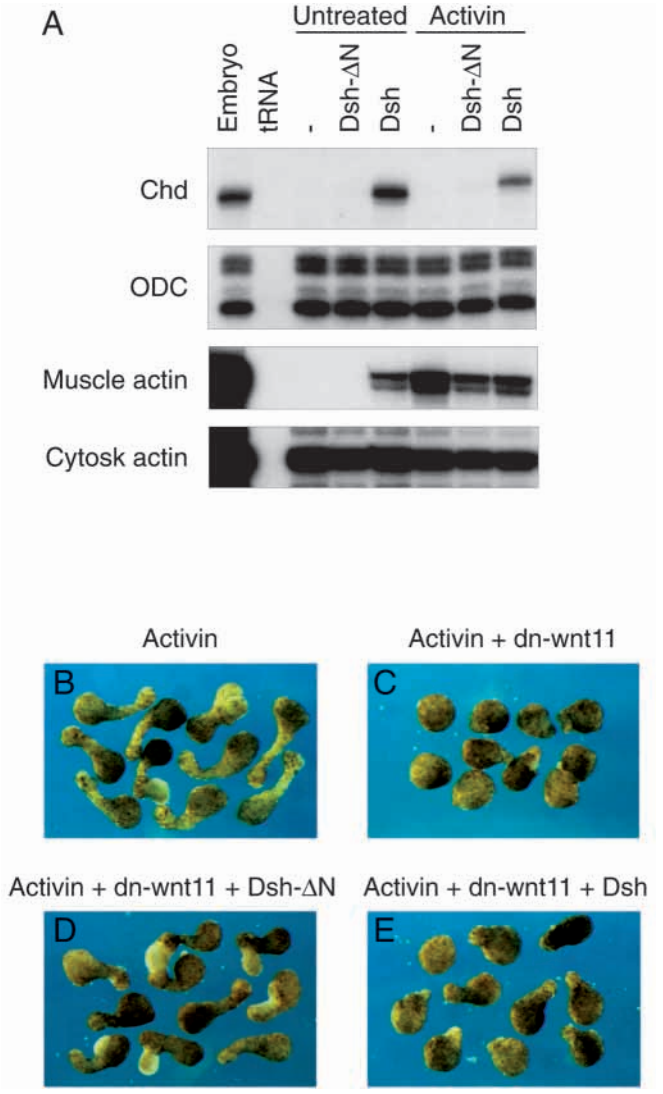

Fig. 10. An N-terminally truncated form of Dsh can rescue the inhibition of convergent extension caused by *dn-wnt11*. (A) Unlike wild-type Dsh, Dsh-ΔN lacks mesoderm-inducing and dorsalising activities. Animal caps derived from embryos injected with 2 ng *Dsh* or 2 ng *Dsh-ΔN* RNA at the 1-cell stage were treated with activin (8 units/ml) at stage 8.5 and cultured until stage 10.5 for analysis of *chordin* (Chd) expression and until stage 23 for analysis of muscle actin expression. Note that *Dsh-ΔN* cannot alone induce expression of *chordin* or muscle-specific actin, and nor can it dorsalise the response to a low level of activin by activating *chordin*. (B-E) Animal caps derived from uninjected embryos (B) or embryos previously injected with 2 ng *dn-wnt11* RNA either alone (C) or in the presence of 2 ng *Dsh-ΔN* RNA (D) or 2 ng *Dsh* RNA (E) were treated with activin (8 units/ml) at stage 10.5 and cultured until stage 18.

Melton, 1993). Secondly, although overexpression of a dominant-negative *Tcf-3* blocks the canonical Wnt signalling pathway as judged by inhibition of axis formation in *Xenopus*, it cannot, in contrast to *dn-wnt11*, inhibit elongation of *Xenopus* animal pole regions in response to activin (Fig. 8F). Third, *Dsh-DEP+* inhibits activin-induced elongation without inhibiting the canonical Wnt pathway (Fig. 9G,I; Table 1). And finally, overexpression of *Dsh-ΔN*, which does not interact with the canonical Wnt signal transduction pathway (Fig. 9H,I), can rescue the inhibitory effects of dn-wnt11 on activin-induced elongation (Fig. 10D) as well as being capable of rescuing the *silberblick*$^{-/-}$ phenotype in zebrafish (Heisenberg et al., 2000). Together, these experiments suggest that although the effects of Xwnt11 are mediated through Dishevelled, subsequent signalling events occur through a β-catenin-independent pathway.

Similarity between morphogenetic movements in *Xenopus* and planar polarity signalling in *Drosophila*

The morphological effects of different Dishevelled constructs (Fig. 9) reveal similarities in the signalling pathways required for convergent extension in *Xenopus* and the establishment of planar polarity in *Drosophila* (reviewed by Boutros and Mlodzik, 1999). In *Drosophila*, mutations in *dsh* cause defects in the orientation of cells within epithelia of the wing, thorax and eye. For example, hairs in the wing usually point distally; the *dsh*^{1} allele causes these hairs to become orientated in a highly abnormal fashion (Adler, 1992). Genetic and biochemical studies show that the 'planar polarity' signalling required to establish correct cellular orientation does not involve components usually placed downstream of Dsh, including Zw-3 (GSK-3), Arm (β-catenin) and Pan (Tcf-3). Rather, it consists of small GTPases such as RhoA and Rac followed by the activation of JNK/SAPK-like kinases (Boutros et al., 1998; Strutt et al., 1997).

The Dsh genes have three conserved domains (Fig. 9A). The N-terminal DIX (*Dis*hevelled-A*xin*) domain is involved in protein-protein interactions and is necessary for the stabilisation of β-catenin (Kishida et al., 1999). The PDZ domain is also involved in protein-protein interactions, and may be involved in recruiting signalling proteins into larger, membrane-associated complexes (Ponting et al., 1997). Finally, the DEP domain (*D*ishevelled-*E*GL10-*P*leckstrin) is thought to be involved in G protein signalling and membrane localisation and also plays a role, perhaps independent of G proteins, in activation of JNK/SAPK-like kinases (Axelrod et al., 1998; Li et al., 1999; Ponting and Bork, 1996).

In *Drosophila*, use of transgenic embryos expressing different domain deletions reveals that the DEP domain is essential for planar polarity signalling, whereas the DIX domain, which is essential for signalling through the canonical Wnt pathway, is not involved (Axelrod et al., 1998; Boutros et al., 1998). Similarly, in our experiments the DEP domain (as well as the PDZ domain) but not the DIX domain is required to restore activin-induced elongation in animal caps expressing dn-wnt11 (Fig. 10).

The similarities in the signalling pathways required for morphogenetic movements in *Xenopus* and the establishment of planar polarity in *Drosophila* raises the intriguing possibility that *Xwnt11* may function to control cell polarity during gastrulation in *Xenopus*. This is discussed below.

The control of gastrulation

Gastrulation involves changes in cell adhesion, cell movement and cell polarity. Our experiments implicate Xwnt11 as a crucial regulator of gastrulation, and it is possible, by analogy with *Drosophila* planar polarity signalling, that its role is to influence cell polarity during convergent extension. We speculate that Xwnt11 activates Dsh which, in turn, regulates

the activity of the Rho family of small GTPases. Such a signalling pathway is required for directing cell shape changes associated with morphogenetic movements in *Drosophila* gastrulation (Barrett et al., 1997), and Rho family members may be involved in similar processes in gastrulation in *Xenopus*. In the future, we plan to investigate the importance of cell polarity during *Xenopus* gastrulation.

Clearly, many questions remain to be answered. In particular, it is necessary to investigate further the Xwnt11 signalling pathway. Recent studies have demonstrated that Xwnt5A, but not Xwnt8, is able to cause the release of intracellular calcium by activating G-protein-linked phosphatidylinositol signalling (Slusarski et al., 1997). This leads to the translocation to the membrane, and activation, of protein kinase C (PKC) (Sheldahl et al., 1999). It is possible that Xwnt11 signals through a pathway similar to that employed by Xwnt5A (see Fig. 4C) and even that phosphorylation of Dsh is regulated by calcium influx and activation of PKC.

Finally, it is necessary to ask why overexpression of wild-type Xwnt-5A, a member of the same class of Wnts as Xwnt11 (Torres et al., 1996), behaves like dn-wnt11 in that it inhibits activin-induced elongation of animal caps (Moon et al., 1993). The simplest explanation is that overexpression of such a Wnt family member causes a loss of cell polarity just as effectively as does its complete absence. Consistent with this suggestion, overexpression in *Drosophila* of fz1, a putative Wnt receptor, leads to a planar polarity phenotype similar to that observed in a loss-of-function mutation (Krasnow and Adler, 1994).

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