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

Masazumi Tada and J. C. Smith*

Division of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK

*Author for correspondence (e-mail: jim@nimr.mrc.ac.uk)

Accepted 1 March; published on WWW 18 April 2000

SUMMARY

Gastrulation in the amphibian embryo is driven by cells of the mesoderm. One of the genes that confers mesodermal identity in Xenopus is Brachury (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 Brachury (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-EnR. Overexpression of a dominant-negative form of Xwnt11, like overexpression of Xbra-EnR, 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, Brachury, 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 Brachury (Xbra), which is expressed in a widespread fashion throughout the mesoderm (Smith et al., 1991), is of some interest. Brachury 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 Brachury 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 Brachury 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 Brachury-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
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 (Sequid-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 BCP.

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 XhoI 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 (Isacs et al., 1992). To make an Xwnt11 probe, pPCR121 was linearised with Hinfl 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

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., 1996). 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'-CCCCCTCAGAGTACCAATGGCTCCGACCCG-3' and 5'-TTGGAGATCTTTCAGCAGTAGTCAGGGGAAC-3'. The PCR product obtained was cloned into the XhoI and BglII 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 pC82-myc. Constructs were as follows: Dsh-ΔN (amino acids 178-736), Dsh-APDZ (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 BamHI and transcribed with SP6 RNA polymerase. All pcS2-dsh constructs were linearised with NotI and transcribed with SP6 RNA polymerase. RNA was synthesised 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 (Sequid-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 BCP.

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 XhoI 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 (Isacs et al., 1992). To make an Xwnt11 probe, pPCR121 was linearised with Hinfl and transcribed with T3 RNA polymerase. Northern blotting was performed as described (Sambrook et al., 1989).
Xwnt11 regulates gastrulation movements 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-GR 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-EnR (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-EnR 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-EnR, 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.
by extension movements (Fig. 7A), while elongation was inhibited by tissue. Control DMZ expressing Xwnt8 and goosecoid affecting expression of the dorsal mesodermal markers affecting notochord differentiation (Fig. 7D) and without and Smith, 1987; Zhong et al., 1999). In previous work, we have

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 allele causes defects in the orientation of ommatidia.

**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 allele causes defects in the orientation of ommatidia.

**Xwnt11 regulates gastrulation movements**

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 pannmesodermal 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-Enk 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.

**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 allele causes defects in the orientation of ommatidia.

**Fig. 4. Dn-wnt11 modulates phosphorylation of Dsh.** (A) Dn-wnt11 downregulates a slow-moving form of Dsh. (Left panel) Animal caps injected with 200 pg mtc-dsh RNA either alone or with 250 pg Xwnt11 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 RNA was sufficient to block elongation of animal caps in response to activin (Fig. 8F; Table 1), while only half the amount of RNA was sufficient to block elongation of animal caps in response to activin, Tcf3 (Fig. 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-Enk 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.

**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 allele causes defects in the orientation of ommatidia.
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-PDZ (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; 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...
**Xwnt11 regulates gastrulation movements**

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-EnR (not shown).

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-EnR 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.

---

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>–</th>
<th>+</th>
<th>++</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>75</td>
</tr>
<tr>
<td>dn-wnt11</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>24</td>
</tr>
<tr>
<td>Dsh-ΔN</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Activin</td>
<td>–</td>
<td>11</td>
<td>89</td>
<td>75</td>
</tr>
<tr>
<td>Activin + Xbra-EnR</td>
<td>98</td>
<td>2</td>
<td>–</td>
<td>48</td>
</tr>
<tr>
<td>Activin + dn-wnt11</td>
<td>89</td>
<td>11</td>
<td>–</td>
<td>59</td>
</tr>
<tr>
<td>Activin + dn-dsh</td>
<td>95</td>
<td>5</td>
<td>–</td>
<td>63</td>
</tr>
<tr>
<td>Activin + ΔN-Te3</td>
<td>–</td>
<td>3</td>
<td>97</td>
<td>39</td>
</tr>
<tr>
<td>Activin + Dsh-ΔN</td>
<td>–</td>
<td>3</td>
<td>97</td>
<td>35</td>
</tr>
<tr>
<td>Activin + Dsh-ΔC</td>
<td>49</td>
<td>23</td>
<td>28</td>
<td>39</td>
</tr>
<tr>
<td>Activin + Dsh-DEP+</td>
<td>53</td>
<td>27</td>
<td>20</td>
<td>51</td>
</tr>
<tr>
<td>Activin + dn-wnt11 + Dsh</td>
<td>15</td>
<td>56</td>
<td>29</td>
<td>39</td>
</tr>
</tbody>
</table>

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

---

**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.
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) sensitize 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
Xwnt11 regulates 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-EnR (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. 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).
Dsh-D with 2 ng derived from uninjected embryos (B) or embryos previously injected to a low level of activin by activating chordin or muscle-specific actin, and nor can it dorsalise the response or 2 ng Dsh activities. Animal caps derived from embryos injected with 2 ng wild-type Dsh, Dsh-D, finally, overexpression of Dsh inhibiting the canonical Wnt pathway (Fig. 9G,I; Table 1). And Xenopus animal pole regions in response to activin (Fig. 8F).

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 dsh1 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 (Dishevelled-Axin) 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 (Dishevelled-EGH10-Pleckstrin) 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 (Sulsarski et al., 1997). This leads to the translocation to the membrane, and activation, of protein kinase C (PKC) (Sheidahl 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 *Iz1*, a putative Wnt receptor, leads to a planar polarity phenotype similar to that observed in a loss-of-function mutation (Krasnow and Adler, 1994).

This work was supported by the Medical Research Council and the Human Frontier Science Program Organisation. We thank Frank Conlon, Derek Stemple and Jean-Paul Vincent for helpful discussion, and Hans Clevers, Doug Melton, Randy Moon and Sergei Sokol for cDNAs.

**REFERENCES**


