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Xwnt11 and the regulation of gastrulation in *Xenopus*

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The molecular basis of gastrulation is poorly understood. In this paper we address this problem by taking advantage of the observation that the transcription activator Brachyury is essential for gastrulation movements in *Xenopus* and mouse embryos. We infer from this observation that amongst the target genes of Brachyury are some that are involved in the regulation of gastrulation. In the course of a screen for Brachyury targets we identified *Xwnt11*. Use of a dominant-negative Xwnt11 construct confirms that signalling by this class of Wnts is essential for normal gastrulation movements, and further investigation suggests that Xwnt11 signals not through the canonical Wnt signalling pathway involving GSK-3 and β-catenin but through another route, which may require small GTPases such as Rho and Rac. Future work will concentrate on elucidating the Xwnt11 signal transduction pathway and on investigating its influence on cell shape and polarity during *Xenopus* gastrulation.

Keywords: morphogenesis; gastrulation; Brachyury; Wnt signalling

1. INTRODUCTION

Although the gastrulation movements of *Xenopus laevis* have been described in great detail and with great insight by Keller and his colleagues (this issue), it remains true that we understand very little about the molecular basis of gastrulation in this species or, indeed, in any other animal. What are the molecular changes that cause bottle cell formation? How do cells change their adhesion properties? Which molecules drive intercalation? What confers polarity? Our ignorance of this fundamental morphogenetic process is quite profound. In this paper we address the question by taking advantage of our knowledge of the transcription factor Brachyury (Herrmann *et al*. 1990; Papaioannou 1997; Smith 1997). Previous work has shown that *Brachyury* function is required for normal gastrulation movements in both *Xenopus* and mouse embryos (Conlon *et al*. 1996; Conlon & Smith 1999; Wilson *et al*. 1995). Brachyury is a transcription activator (Conlon *et al*. 1996; Kispert *et al*. 1995*a*), and this suggests that its downstream targets include genes that are involved in the control of gastrulation. A search for such targets has identified *Xwnt11*, a member of the Wnt family of secreted signalling molecules (Ku & Melton 1993). Our experiments show that *Xwnt11*, like *Brachyury*, is required for normal gastrulation movements in *Xenopus*, and they suggest that Xwnt11 does not act through the canonical Wnt signalling pathway involving GSK-3 and β-catenin (Cadigan & Nusse 1997) but through a pathway similar to that involved in planar polarity signalling in *Drosophila* (Axelrod *et al*. 1998; Boutros & Mlodzik 1999; Boutros *et al*. 1998). It is possible, therefore, that Xwnt11 signalling functions to control cell polarity during gastrulation in *Xenopus*, perhaps by regulating the function of small GTPases of the Rho family.

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2. BRACHYURY

The *Brachyury* (or *T*) gene was first identified over 70 years ago. Mice that are heterozygous for the *Brachyury* mutation have a short tail, while homozygous mutant embryos do not form a proper allantois or notochord and lack mesoderm posterior to somite 7 (Chesley 1935; Dobrovolskaïa-Zavadskaïa 1927; Gluecksohn-Schoenheimer 1944). This dramatic mutant phenotype indicated that *Brachyury* plays a key role in the formation of mesodermal structures, but significant progress in the analysis of *Brachyury* function only came with the cloning of the gene by Herrmann *et al*. (1990). This work revealed that *Brachyury* encodes a protein of 436 amino acids and that the gene is expressed at the highest levels, and for the longest times, in those structures that are absent in homozygous mutant embryos (Wilkinson *et al*. 1990). Thus transcripts are present initially throughout the primitive streak of the embryo, and expression is maintained in the notochord and tailbud.

Homologues of *Brachyury* were identified in other vertebrate species including chicken, *Xenopus* and zebrafish (Kispert *et al*. 1995*b*; Schulte-Merker *et al*. 1992; Smith *et al*. 1991). All proved to have similar expression patterns, and experiments in these species emphasized the importance of *Brachyury* in mesoderm formation. The *no tail* mutation in the zebrafish, for example, proved to encode a fish homologue of *Brachyury* (Schulte-Merker *et al*. 1994), and misexpression of *Xenopus Brachyury* (*Xbra*) in prospective ectoderm of the early gastrula proved to be sufficient to induce ectopic mesoderm of ventral and posterior character (Cunliffe & Smith 1992, 1994; O'Reilly *et al*. 1995). *Brachyury* is therefore both necessary

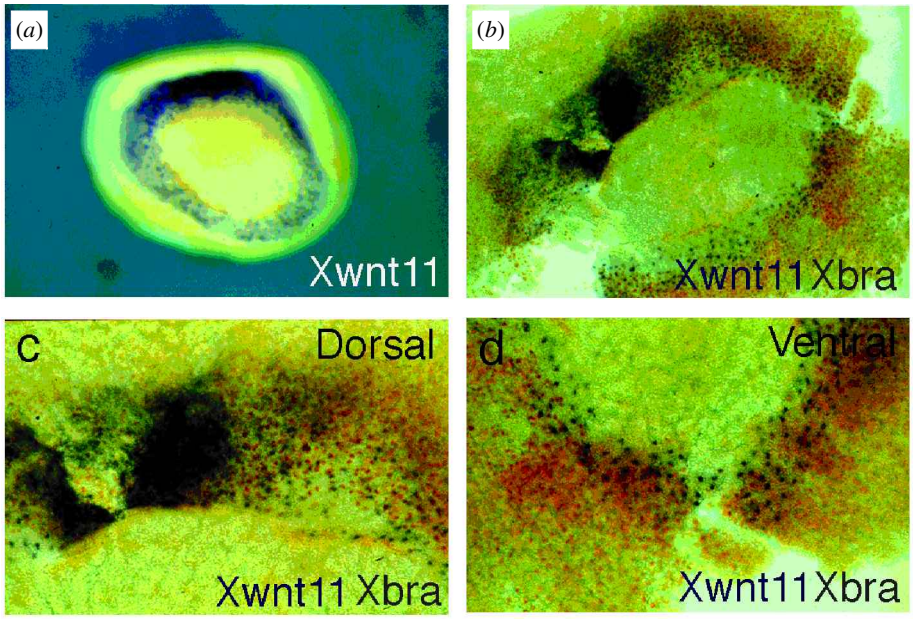

Figure 1. Comparison of the expression patterns of *Xwnt11* and Xbra at the early gastrula stage. (*a*) *In situ* hybridization showing expression of *Xwnt11* in the early gastrula. (*b*, *c*) Flat-mount preparations showing *Xwnt11* RNA expression (blue), revealed by *in situ* hybridization, and Xbra protein (brown) revealed by antibody staining. (*b*) View showing whole marginal zone (prospective mesoderm). (*c*) Dorsal view at higher power. (*d*) Ventral view at higher power.

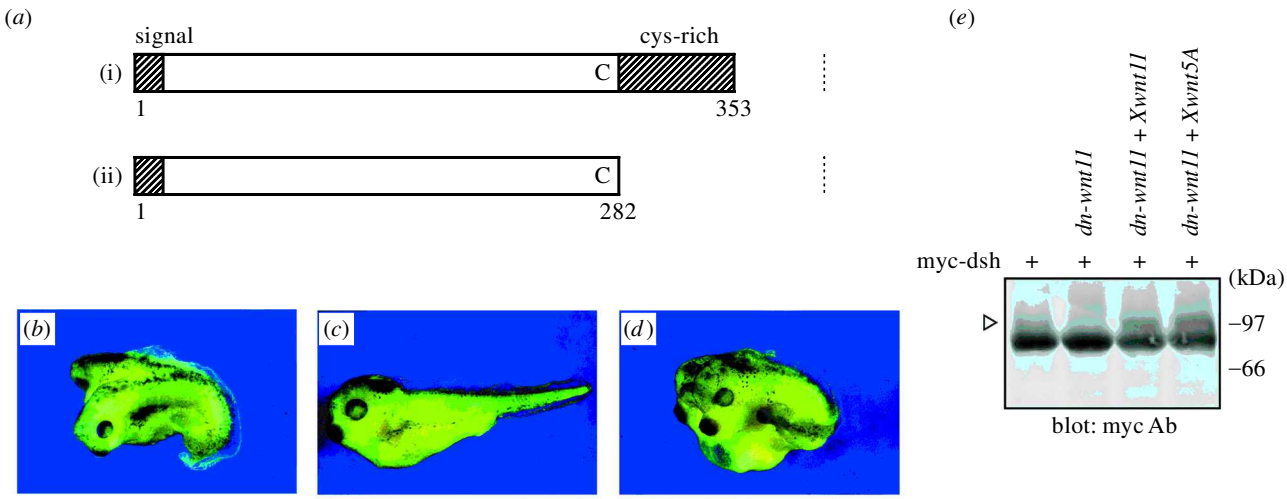

Figure 2. Specificity of dominant-negative Xwnt11. (*a*) Schematic illustrations of (i) wild-type Xwnt11 and (ii) dn-wnt11, which carries a C-terminal truncation. (*b–d*) *Xenopus* embryos were injected with 2pg *Xwnt8* RNA either alone (*b*) or in the presence of a 20-fold excess of *dn-wnt8* RNA (*c*) or *dn-wnt11* RNA (*d*). Note that axis duplication caused by Xwnt8 is inhibited by *dn-wnt8* but not by *dn-wnt11*. (*e*) A hyperphosphorylated form of myc-Dsh (triangle) present in uninjected animal caps is downregulated by dn-wnt11 and rescued by overexpression of *Xwnt11* and *Xwnt5A* RNA.

and sufficient for the formation at least of posterior mesoderm.

Investigation of the Brachyury protein showed that the N-terminal half of the protein has sequence-specific DNA-binding activity, with polymerase chain reaction-based binding-site selection experiments identifying the partially palindromic sequence T[G/C]ACACCTAGG TGTGAAATT (Kispert & Herrmann 1993). Crystallographic studies indicated that Brachyury binds such a sequence as a dimer, and that it binds in a novel manner in which a C-terminal helix becomes embedded into an enlarged minor groove of DNA without causing bending (Muller & Herrmann 1997). More recent experiments, however, show that Brachyury can also bind a half-palindromic sequence such as [T/C]TTCACACCT (Casey *et al*. 1998, 1999; Tada *et al*. 1998), and it will be interesting to investigate the mode of Brachyury binding under these conditions.

The C-terminal half of Brachyury functions as a transcription activator (Conlon *et al*. 1996; Kispert *et al*. 1995*a*), and experiments in *Xenopus* show that the main function of Brachyury is indeed to activate the expression of downstream genes; expression of an interfering construct in which the activation domain of Xbra is

replaced by the repressor domain of *Drosophila* Engrailed (Xbra-En^R) causes embryos to lose posterior structures and to exhibit deficiencies in notochord differentiation (Conlon *et al*. 1996). This phenotype resembles that of *Brachyury* and *no tail* homozygous mutant embryos.

We now know that Brachyury is the founder member of a family of proteins that share sequence homology with the N-terminal DNA-binding domain described above—the so-called 'T-box' (reviewed by Papaioannou & Silver 1998; Smith 1997, 1999). The first protein to be recognized as being homologous to Brachyury was *Drosophila* optomotor-blind (Pflugfelder *et al*. 1992); since then the family has expanded to include at least seven T-box proteins in the mouse (not including Brachyury itself) as well as members in vertebrates, ascidians, sea urchin, *Drosophila* and *Caenorhabditis elegans*. The genes are expressed at various stages of development and in various tissues, and they have diverse functions. *Tbx5*, for example, plays a role in heart and forelimb development, while *Tbx4* regulates hindlimb development (Basson *et al*. 1997; Li *et al*. 1997; Logan & Tabin 1999; Rodriguez-Esteban *et al*. 1999; Takeuchi *et al*. 1999).

3. BRACHYURY IS REQUIRED FOR NORMAL GASTRULATION MOVEMENTS

In mouse, fish and frog embryos, lack of *Brachyury* function results in loss of posterior mesodermal tissues and lack of a properly differentiated notochord. At least in mouse and *Xenopus* embryos, this phenotype is presaged by abnormal gastrulation movements. In the mouse, for example, morphogenesis of the primitive streak and notochord is abnormal and there is a failure of the axis to elongate properly. The generation of chimeras containing both wild-type cells and cells lacking *Brachyury* function reveals that the mutant cells accumulate in the primitive streak during gastrulation, leading to their eventual accumulation in the tailbud (Wilson *et al*. 1995). This suggests that one role of Brachyury might be to alter the adhesion properties of cells as they pass through the primitive streak.

Similar defects in gastrulation movements are observed in *Xenopus* embryos in which Brachyury function is inhibited by the interfering construct Xbra-En^R. In particular, the blastopore is misshapen and slow to close, as if convergent extension movements are compromised (Conlon *et al*. 1996). The clearest illustration of the requirement for *Brachyury* during gastrulation, however, comes from studies on *Xenopus* animal pole regions. 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 (reviewed by Harland & Gerhart 1997). Candidates for the mesoderm-inducing signals include members of the transforming growth factor-β family of growth and differentiation factors, such as activin, derrière and the nodal-related genes (Harland & Gerhart 1997; Osada & Wright 1999; Sun *et al*. 1999). For example, treatment of prospective ectodermal tissue of the animal pole region with activin causes those cells to form mesodermal cell types. An earlier response to activin, however, is that the animal pole cells undergo coordinated convergent extension movements such that the isolated tissue extends in a dramatic fashion (Symes & Smith 1987). Untreated animal pole regions, by contrast, form spheres of ectodermal cells. The elongation of animal pole regions in response to activin provides a powerful model system for the analysis of gastrulation and convergent extension, and significantly, we find that this elongation does not occur in animal caps derived from embryos injected with RNA encoding Xbra-En^R (Conlon & Smith 1999). Together with the phenotypes of intact *Xenopus* embryos expressing Xbra-En^R, in which gastrulation is impaired, and the results obtained with mouse embryos, these experiments indicate that Brachyury function is essential for gastrulation movements and in particular for convergent extension. Since Brachyury functions as a transcription activator, it is likely that its target genes include some which are required for convergent extension.

4. A SCREEN FOR BRACHYURY TARGETS IDENTIFIES *Xwnt11*

In an effort to identify the target genes of Brachyury, we have screened cDNA libraries in which Brachyury-inducible genes are highly enriched (Saka *et al*. 2000; Tada *et al*. 1998). Among other genes, this screen has allowed us to isolate *Xwnt11*, which was originally identified as a maternally expressed *Wnt* gene whose transcripts are restricted to the vegetal hemisphere of the oocyte and early embryo (Ku & Melton 1993). Zygotic expression of *Xwnt11* proved to resemble closely that of *Xbra*, with expression commencing throughout the marginal zone and persisting in posterior circumblastoporal tissue throughout gastrula stages to the early neurula stage (figure 1) (Saka *et al*. 2000; Tada & Smith 2000).

Two experiments suggest that *Xwnt11* is a direct target of Xbra and that expression of *Xwnt11* in the early embryo requires Xbra function. First, induction of *Xwnt11* by the hormone-inducible construct *Xbra-GR* can occur in the presence of cycloheximide, an inhibitor of protein synthesis (Saka *et al*. 2000). This indicates that induction of *Xwnt11* by Xbra does not require intervening protein synthesis, suggesting that Xbra acts directly on the *Xwnt11* promoter. This issue is now under investigation. Second, expression of the interfering construct Xbra-En^R causes an almost complete downregulation of *Xwnt11* in the *Xenopus* embryo, arguing that Xbra function is required for expression of *Xwnt11* (Tada & Smith 2000).

5. Xwnt11, LIKE Xbra, IS REQUIRED FOR GASTRULATION MOVEMENTS

(a) *Specificity of a dominant-negative Wnt11 construct*

To investigate the function of *Xwnt11* during gastrulation, we constructed a C-terminally truncated form of the protein (dn-wnt11) which, by analogy with a similar *Xwnt8* construct (Hoppler *et al*. 1996), might be expected to act in a dominant-negative fashion (figure 2*a*). Wnt11 and Wnt8 belong to different classes of the Wnt family (Du *et al*. 1995), and it is important to demonstrate first that our dn-wnt11 construct does indeed inhibit the function of Wnt11 (and perhaps other members of the same class such as Wnt5) and second that it has no effect on the activity of the Wnt8 class.

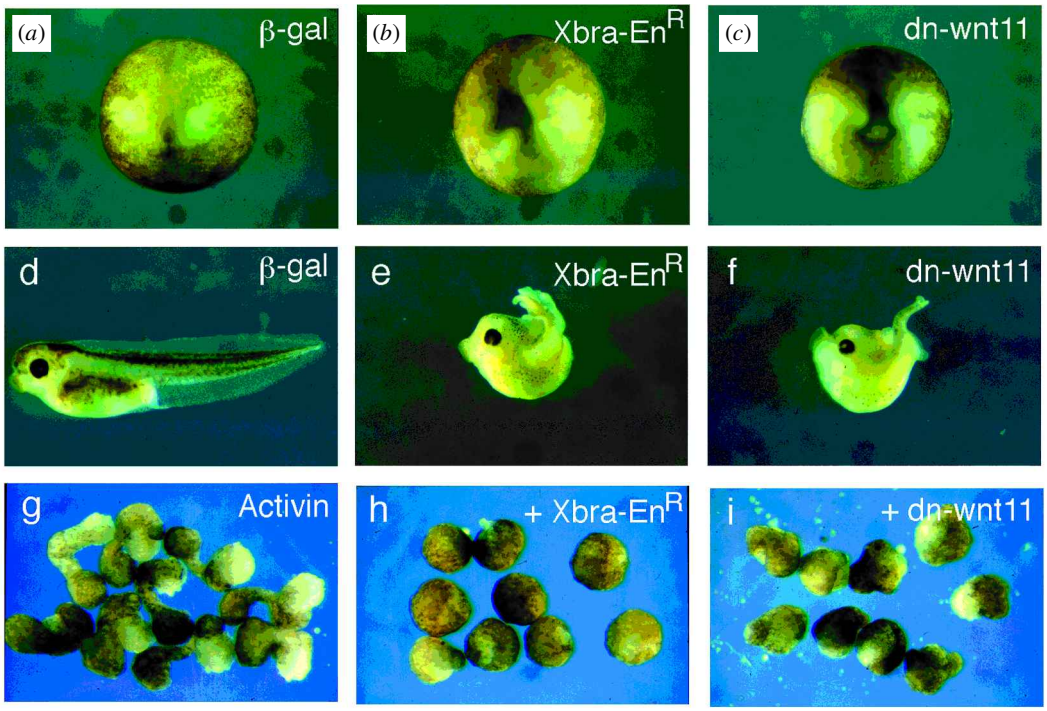

Figure 3. Xwnt11, like Xbra, is required for gastrulation movements in *Xenopus*. (*a–c*) *Xenopus* embryos at the late gastrula–early neurula stage following injection with RNA encoding (*a*) β-galactosidase as a control, (*b*) Xbra-En^R, and (*c*) dn-wnt11. Note the disruption of gastrulation in (*b*) and (*c*). (*d–f*) *Xenopus* embryos at tadpole stages after injection with RNA encoding (*d*) β-galactosidase, (*e*) Xbra-En^R, and (*f*) dn-wnt11. Note loss of posterior structures in (*e*) and (*f*). (*g–i*) Activin-treated animal pole regions derived from uninjected embryos (*g*) or embryos injected with RNA encoding Xbra-En^R (*h*) or dn-wnt11 (*i*). Note inhibition of elongation in (*h*) and (*i*).

To confirm that dn-wnt11 does indeed inhibit the function of wild-type members of this Wnt class, we isolated animal caps from embryos expressing a myc-tagged form of Dishevelled ((Dsh), for review see Boutros & Mlodzik 1999). Western blotting revealed the presence of a hyperphosphorylated form of Dsh in such animal caps, which may be due to high levels of signalling by Xwnt5A, which is expressed in this region (Morgan *et al*. 1999). This hyperphosphorylated form of Dsh was downregulated by dn-wnt11, but could be 'rescued' by co-expression of wild-type Xwnt11 or Xwnt5A (figure 2*e*). Together, these experiments indicate that dn-wnt11 inhibits the function of the class of Wnts which includes Wnt5A and Wnt11.

By contrast, dn-wnt11 appears not to inhibit the function of the other class of Wnts, which includes Wnt1 and Wnt8. This was examined using an axis-induction assay, in which injection of RNA encoding Xwnt8 into the *Xenopus* egg causes complete axis duplication (McMahon & Moon 1989; Smith & Harland 1991; Sokol *et al*. 1991). This duplication can be inhibited by dominant-negative Xwnt8, but not by similar concentrations of dn-wnt11 (figure 2*b–d*).

Together, these experiments suggest that dn-wnt11 inhibits the function of Wnt11 itself, and of Wnt5A, but does not affect the action of Wnt1 or Wnt8. With this information in hand, we went on to investigate the effects of dn-wnt11 on gastrulation.

(b) *dn-wnt11 blocks gastrulation movements*

Expression of *dn-wnt11* throughout the *Xenopus* embryo caused embryos to lack posterior structures, but differentiation of muscle and notochord in anterior regions occurred normally (figure 3*d–f* and data not shown). At earlier stages, expression of *dn-wnt11* resulted in delayed and incomplete gastrulation movements, reminiscent of those caused by *Xbra-En^R* (figure 3*a–c*). However, the expression of a variety of mesodermal markers, including *Xbra*, *goosecoid* and *Myf-5*, were unaffected, suggesting that dn-wnt11 inhibits gastrulation without affecting mesodermal specification or differentiation (Tada & Smith 2000).

These conclusions were confirmed by experiments in activin-treated animal caps; dn-wnt11 prevented activin-induced elongation of isolated animal pole regions (figure 3*g–i*), but had no effect on the ability of activin to induce expression of the mesodermal markers *Xbra*, *goosecoid*, *Xwnt8* or *Bix1* (Tada & Smith 2000). Thus, inhibition of signalling by the class of Wnt signalling molecules that includes Wnt11 prevents normal gastrulation movements, but does not affect mesodermal specification.

6. Xwnt11 DOES NOT ACT THROUGH THE CANONICAL Wnt SIGNALLING PATHWAY

What is the intracellular signalling pathway employed by Xwnt11? The ability of Xwnt11 to regulate the hyperphosphorylation of Dsh suggests that it might act through the canonical Wnt pathway involving Dsh, GSK-3 and β-catenin (Cadigan & Nusse 1997), and in support of this idea we find that Dsh can 'rescue' the inhibition of activin-induced elongation of animal caps that is caused by dn-wnt11 (figure 4*e*). To investigate this question further, we made use of ΔN-Tcf3, a dominant-negative version of Tcf3 (T-cell factor 3) which can block the

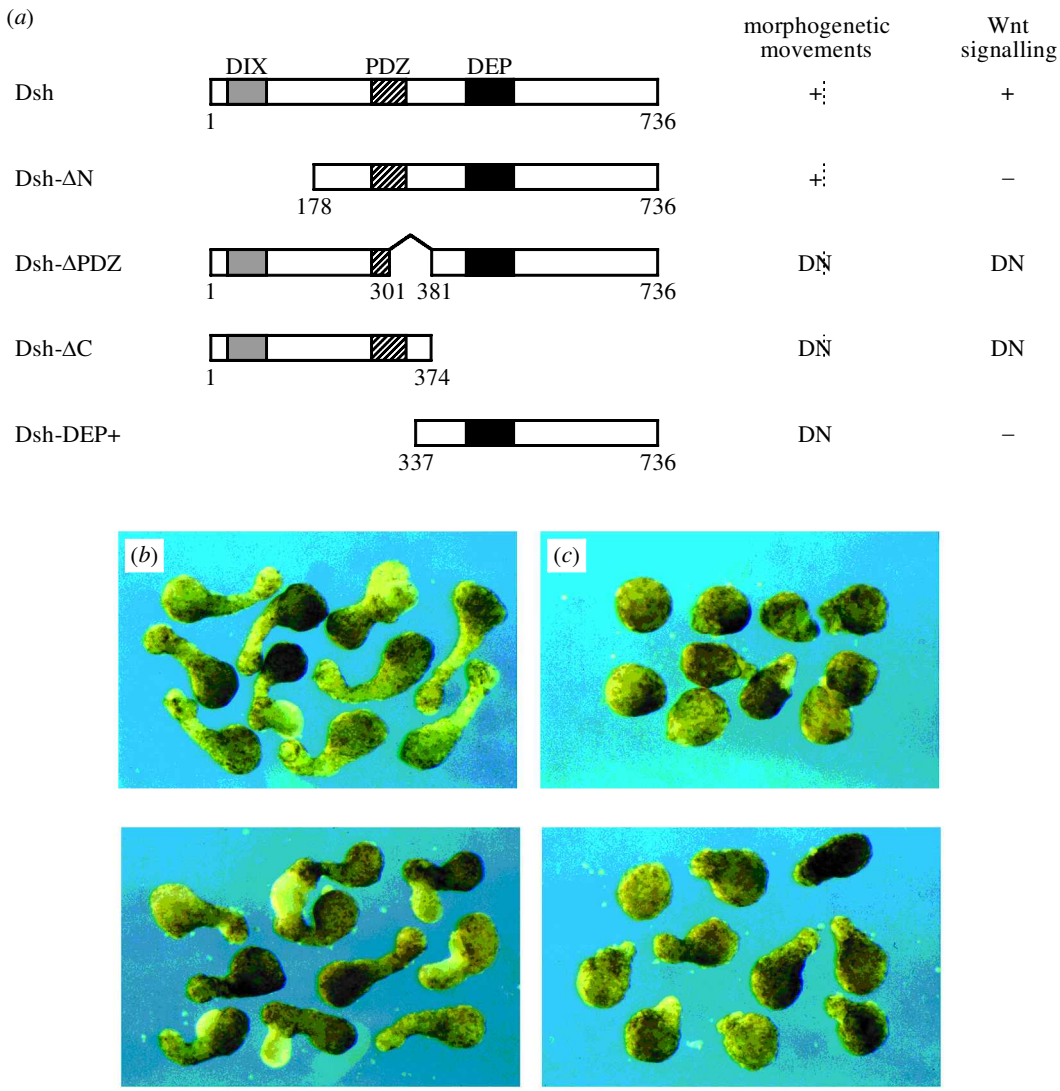

Figure 4. Mapping of Dsh domains required for convergent extension movements. (*a*) Different Dsh constructs used in our experiments. DN, dominant-negative. Effects of each construct are based on experiments described in this paper, and by Tada & Smith (2000), Axelrod *et al.* (1998), Boutros & Mlodzik (1999) and Boutros *et al.* (1998). (*b–e*) Dsh and Dsh-ΔN can rescue the inhibition of activin-induced elongation of animal caps caused by *dn-wnt11*. (*b*) Activin induces elongation of untreated animal caps. (*c*) Elongation is inhibited by *dn-wnt11*. (*d*, *e*) Rescue of elongation by (*d*) Dsh-ΔN (activin+dnwnt11+Dsh-ΔN) and (*e*) Dsh (activin+dnwnt11+Dsn).

transcriptional function of β-catenin (Molenaar *et al.* 1996). Surprisingly, ΔN-Tcf3 did not block activin-induced elongation of animal caps (Tada & Smith 2000), suggesting that in the regulation of gastrulation, Xwnt11 signalling 'branches' from the canonical Wnt signalling pathway somewhere downstream of Dsh.

(a) *Mapping domains of Dsh*

The idea that separate signalling pathways derive from Dsh is consistent with genetic evidence in *Drosophila*. The first *dishevelled* mutant to be isolated, dsh^1, causes defects in the polarity of bristles on the wing and thorax, a phenotype that gave the mutant its name (Fahmy & Fahmy 1959). Additional genetic screens, however, isolated null alleles of *dsh* the phenotypes of which were completely different, and resembled those of *wingless* (a *Drosophila* Wnt family member) and *armadillo* (*Drosophila* β-catenin) (Nüsslein-Volhard & Wieschaus 1980; Perrimon & Mahowald 1987). Molecular characterization of Dsh suggests that the different phenotypes are the consequences of mutations occurring in different domains of the protein (Axelrod *et al.* 1998; Boutros & Mlodzik 1999; Boutros *et al.* 1998; and see figure 4*a*). Thus, the DEP domain of Dsh proves to be essential for polarity signalling, whereas the DIX domain is dispensable. In contrast, the DIX domain is essential for signalling through β-catenin, but the DEP region is not required. Consistent with these conclusions, the original dsh^1 allele has a single amino-acid substitution in a conserved region of the DEP domain.

(b) *Xwnt11 acts through the 'polarity' pathway*

We have taken advantage of this information to ask whether Xwnt11 signals through the β-catenin pathway or the polarity pathway. Consistent with the suggestion above that Xwnt11 signalling branches from the canonical Wnt signalling pathway, we find that Dsh constructs lacking just the DIX domain are able to rescue the

inhibition of activin-induced elongation of animal caps that is caused by dn-wnt11 (figure 4*d*). This result suggests that Xwnt11 regulates gastrulation through a pathway analogous to that used in polarity signalling in the *Drosophila* embryo. This is discussed below (§7(c, d)).

7. DISCUSSION AND CONCLUSIONS

In an attempt to understand the molecular basis of gastrulation, we have taken advantage of the observation that loss of *Brachyury* function causes a severe disruption of convergent extension. Since Brachyury is a transcription activator, and the ability of Brachyury to activate transcription is essential for its function, we deduced that amongst its target genes there must be some involved in the regulation of gastrulation. *Xwnt11* proved to be such a gene.

(a) *Xwnt11 as a target of Brachyury*

Our experiments suggest that *Xwnt11* is a direct target of Xbra and that Xbra function is essential for expression of *Xwnt11* during early *Xenopus* development. Confirmation that *Xwnt11* is a bona fide target of Xbra, however, awaits analysis of the *Xwnt11* 5′ regulatory region, where we hope at least to identify Brachyury 'half-sites', as are present in the *eFGF* and *Bix4* promoters, both of which are direct targets of T-box genes (Casey *et al*. 1998, 1999; Tada *et al*. 1998).

(b) *Xwnt11 function is required for normal gastrulation movements*

The only practical way to study gene function in *Xenopus*, at least for now, is to make use of antisense technology or dominant-negative constructs. To this end we have made a dominant-negative version of Xwnt11, and demonstrated that it inhibits the function of the Wnt5A/Wnt11 class of Wnts, but not the Wnt1/Wnt8 class. Expression of dn-wnt11 in the *Xenopus* embryo interferes with gastrulation movements, and it also interferes with the elongation of isolated dorsal marginal zone regions and activin-treated animal caps.

These experiments are consistent with the idea that Xwnt11 function is required for normal gastrulation movements, but it is possible, of course, that dn-wnt11 also interferes with the function of other members of the Wnt11 class of Wnts, such as Xwnt5A. Of the known members of this class, however, only Wnt11 is expressed in the mesoderm of the embryo during gastrulation (Du *et al*. 1995; Moon *et al*. 1993; Morgan *et al*. 1999), suggesting that the effects of dn-wnt11 are indeed due to interference of the function of this family member.

(c) *Wnt11 does not signal through the canonical Wnt pathway*

Experiments using a dominant-negative version of Tcf3, and others using different domains of Dsh, suggest that Xwnt11 does not signal through the canonical Wnt signalling pathway involving Dsh, GSK-3 and β-catenin. Rather, it may use a pathway more reminiscent of that used in polarity signalling during *Drosophila* development. This pathway is less well understood than the canonical pathway, but involves small GTPases such as RhoA and Rac followed by the activation of JNK/SAPK-like kinases (Boutros & Mlodzik 1999; Boutros *et al*. 1998; Strutt *et al*. 1997). In this regard it is interesting that these components have been implicated in migration and cell shape changes occurring during gastrulation and dorsal closure in *Drosophila* (Barrett *et al*. 1997; Noselli & Agnes 1999), and it is an intriguing possibility that the same intracellular signalling pathways regulate gastrulation in both vertebrates and invertebrates.

(d) *The future*

Our work raises more questions than it answers. One task, as suggested above, is to investigate further the Xwnt11 signalling pathway. As well as the possible involvement of Rho- and Rac-like molecules, it is important to note that Xwnt5A, but not Xwnt8, can cause the release of intracellular calcium via G-protein-linked phosphatidylinositol signalling (Slusarski *et al*. 1997), and thereby activate protein kinase C (Sheldahl *et al*. 1999). It is possible that Xwnt11 employs a similar pathway. One should also remember that thinking of Wnt signalling pathways as being linear may be a mistake—a model based on a signalling network may be more appropriate (Martinez Arias *et al*. 1999).

At the embryological level it may be helpful to extend the analogy with polarity signalling in *Drosophila* to look at the cell polarity during *Xenopus* gastrulation, and to ask if polarity is disrupted by inhibition of Xwnt11 signalling. Along these lines, it may be significant that gastrulation movements in *Xenopus* are not only disrupted by inhibiting signalling by this class of Wnt—overexpression of Xwnt5A also blocks convergent extension (Moon *et al*. 1993). This may reflect the fact that cell polarity can in principle be disrupted both by removing localized Wnt signalling and also by completely flooding the system. We note that in *Drosophila*, overexpression of the Wnt receptor frizzled 1 causes a polarity phenotype similar to that observed in a loss-of-function mutation (Krasnow & Adler 1994).

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