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Xbra functions as a switch between cell migration and convergent extension in the *Xenopus* gastrula

Kristen M. Kwan and Marc W. Kirschner

Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (e-mail: marc@hms.harvard.edu)

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SUMMARY

During *Xenopus* gastrulation, the dorsal mesoderm exhibits two different cell behaviors in two different regions: active cell migration of prechordal mesoderm and convergent extension of chordamesoderm. Although many genes involved in specification and differentiation of the dorsal mesoderm have been studied, the role of these genes in controlling cell behaviors is poorly understood. To understand better the link between the development and cell behaviors of the dorsal mesoderm, we have examined these behaviors in dissociated cells and explants, where activin protein can induce both active cell migration and convergent extension. We find that *Xbra*, a transcription factor necessary for convergent extension, actively inhibits cell migration, both in animal cap explant assays and in the endogenous dorsal mesoderm. In addition, *Xbra* appears to inhibit cell migration by inhibiting adhesion to fibronectin. We propose that *Xbra* functions as a switch to keep cell migration and convergent extension as mutually exclusive behaviors during gastrulation.

Key words: *Xenopus*, Gastrulation, *Xbra*, Cell migration, Convergent extension, Wnt

INTRODUCTION

Gastrulation is the process by which the basic body plan of the embryo is established from the provisional geometry of the egg. A concerted set of cell movements rearranges the germ layers to their final positions relative to one another, with endoderm on the inside, ectoderm on the outside, and mesoderm between the two. In *Xenopus* embryos, the Spemann organizer, or the upper dorsal blastopore lip, is crucial for the generation of the basic body plan. The anterior region of the Spemann organizer involutes first and forms prechordal mesoderm, while the later involuting region forms chordamesoderm and gives rise to the notochord. These regions can be distinguished by their early gene expression and type of cell motility during gastrulation, as well as their respective fates. Prechordal mesoderm cells exhibit active cell migration, while cells in the chordamesoderm undergo convergent extension. These two behaviors have been studied extensively on a descriptive level, particularly in *Xenopus* (Gerhart and Keller, 1986; Winklbauer, 1990; Keller and Winklbauer, 1992; Winklbauer and Selchow, 1992; Keller et al., 2000).

Active cell migration of the prechordal mesoderm is characterized by the ability of single cells to spread and crawl upon a fibronectin substrate. During gastrulation, the cells generate bipolar, actin-rich lamellipodia and actively migrate upon the extracellular matrix secreted by the blastocoel roof. Interaction with a fibronectin substrate is necessary, as blockade of cell surface interactions with fibronectin, using either peptides that mimic fibronectin (GRGDSP) or fibronectin-blocking antibodies, abolishes migration (Winklbauer, 1990). Prechordal mesoderm cells migrate in vitro at the same rate as they do in the embryo, about 2.5 μm/minute (Wacker et al., 1998; Winklbauer, 1990). Migrating cells come to rest at the future anterior end of the embryo, where they play a crucial role in patterning the anterior neurectoderm (Sive et al., 1989). Inhibition of active cell migration of the prechordal mesoderm, as caused by fibronectin-blocking antibodies, can lead to a variety of head defects (Marsden and DeSimone, 2001).

In contrast to prechordal mesoderm cell migration, convergent extension is not so much a migrating process as a cell sorting process. Groups of cells rearrange and intercalate to change the overall shape of a tissue. During gastrulation, chordamesoderm cells rearrange from a largely isotropic organization, intercalate and generate a rod-like structure that will differentiate into notochord. Cell-cell adhesion, rather than cell-substrate adhesion involving fibronectin, is crucial for this process. Unlike the case of active cell migration, peptides that mimic fibronectin (GRGDSP) have no effect on the convergence and extension movements of isolated explants (Ramos and DeSimone, 1996; Ramos et al., 1996; Winklbauer and Keller, 1996). Cell-cell adhesion molecules such as cadherins and protocadherins are important for convergent extension. Because cell-cell contact is required for convergent extension, this behavior cannot be observed in isolated single cells, rather, it is analyzed in the context of an intact explant. Defects in this process result in shortened trunks with a fully formed head (Wallingford and Harland, 2001).

These two behaviors are discrete and non-overlapping.

Prechordal mesoderm exhibits active cell migration, but not convergent extension; conversely, chordamesoderm undergoes convergent extension, while only a few cells dissociated from the chordamesoderm actively migrate on fibronectin (Wacker et al., 1998). Gastrulation in *Xenopus laevis* probably presents the most accessible system to study control of these fundamental processes of cell and tissue morphogenesis. Though many growth factors, transcription factors and signaling molecules have been identified in the process of cytodifferentiation, the control of cell behavior and cell motility is not well understood.

To understand better each cell behavior from both a developmental and cell biological standpoint, we have examined embryonic tissues in explants. The animal cap, a tissue explant whose unperturbed fate is ventral ectoderm, can be induced to express prechordal or chordamesoderm markers via treatment with the TGFβ factor activin (Symes et al., 1994; Gurdon et al., 1996; Gurdon et al., 1999). Depending on the conditions of the treatment, either migration or convergent extension can be induced. We have used this system to study factors that are important in the induction or regulation of either behavior.

VegT, a transcription factor of the T-box family, is necessary for both cell behaviors. By contrast, *Xbra*, another T-box transcription factor, although required for convergent extension, inhibits cell migration. This cell migration block can be partially rescued by inhibiting convergent extension downstream of *Xbra*. In addition, *Xbra* appears to inhibit cell migration by specifically inhibiting adhesion to fibronectin. We propose that *Xbra* functions as a fundamental switch to keep cell migration and convergent extension as mutually exclusive behaviors in adjacent domains during gastrulation.

MATERIALS AND METHODS

Xenopus methods

Xenopus embryos were obtained from *X. laevis* frogs (NASCO). They were fertilized by in vitro fertilization, dejellied and cultured at 14-18°C in 0.1× Marc's Modified Ringer's (MMR) (Peng, 1991). Embryos were staged according to (Nieuwkoop and Faber, 1967). For injection with RNAs and DNAs, embryos were placed in 0.1× MMR containing 5% Ficoll and 50 μg/ml gentamycin. Embryos were cultured in 0.1× MMR containing 50 μg/ml gentamycin at 14-18°C until they reached the desired stage. For animal cap explants, both cells of a two-cell embryo were injected superficially in the animal hemisphere. All injection amounts reported are per blastomere.

Prechordal and chordamesoderm explant preparation: at stage 10, the vitelline was removed and incisions made on either side of the dorsal blastopore lip. A third incision was made at the blastopore lip, leaving an explant of involuted prechordal mesoderm, chordamesoderm (preinvolution) and neural tissue. Involuted prechordal mesoderm was lifted off of the explant as an intact piece by inserting an eyebrow knife into Brachet's cleft. The remaining explant was trimmed to remove neural tissue, leaving a chordamesoderm explant. For the mesendoderm extension assay, explants were prepared as described (Davidson et al., 2002).

Activin-induced convergent extension and cell migration assays

Sibling animal caps were dissected at stage 9. Half of the caps were left intact and treated with activin protein for one hour (1 U/ml in 1×MMR). They were then transferred from activin solution into 1×MMR and allowed to heal and grow until stage 19, when they were scored for convergent extension. The rest of the caps were dissociated in CMFM (Ca^{2+} and Mg^{2+} free medium) and then treated with activin protein (1 U/ml in 1×CMFM) for 1 hour. The dissociated cells were subsequently plated in Modified Barth's Solution (MBS) into fibronectin-coated chambered coverslips (VWR). Coverslips were coated with 0.1 mg/ml fibronectin (Sigma, diluted to the appropriate concentration with MBS) for 2 hours at room temperature, and then blocked with bovine serum albumin (BSA; 50 mg/ml in MBS).

For the migration assay, cells were analyzed in the following manner: a field of cells was randomly chosen, and images were captured at 30 second intervals for at least 20 minutes (15 minutes for control samples: uninjected/untreated cells and uninjected/Activin-treated cells). Images were assembled into timelapse form (using Openlab software) and played back at a speed of either 10 or 20 frames per second. A cell was scored as positive for migration if it both translocated across the substrate and exhibited active protrusions.

Image analysis

Timelapse analysis of dissociated cells was performed using a Hamamatsu C2400 CCD Camera attached to a Zeiss Axiovert 135. Images were acquired and timelapse files assembled using Openlab software (Improvision). Fluorescence images were acquired using a Princeton Instruments cooled CCD Camera. Color images were acquired with a Sony 3CCD Color Camera mounted onto a Zeiss Stemi SV11 Stereoscope.

RNAs/DNAs

Plasmid DNAs were linearized overnight and purified using the Qiagen PCR Purification Kit. All RNAs were synthesized using the mMessage mMachine kit (Ambion) for capped RNA, purified using the Qiagen RNeasy Mini Kit, and subsequently precipitated in ethanol and resuspended in RNAse-free water.

*Xbra*ΔDNABD is a delection in amino acids 206-229 (Kispert and Hermann, 1993), *Xdsh* mutants are all as in (Rothbacher et al., 2000). VegT-EnR was constructed as described previously (Horb and Thomsen, 1997). Xbra-EnR was constructed as described previously (Conlon et al., 1996). dn Wnt11 was constructed as previously (Tada and Smith, 2000).

All constructs are in a pCS2 backbone (Rupp et al., 1994; Turner and Weintraub, 1994), except for *Xenopus* Activin, which is in pSP64T.

Activin protein

Xenopus oocytes were harvested by manual defolliculation, and injected with 30 ng of capped RNA encoding full-length *Xenopus* activin. Oocytes were cultured in OR2 solution for oocyte storage (+ 0.5 mg/ml BSA) in 96-well plates for 2 days at 18°C (five oocytes per well in 200 μl culture medium). The supernatant was collected, filtered, aliquotted and stored at –80ºC. Activity of each batch was tested, and was consistently found to be 20 U/ml [units as defined by Green et al. (Green et al., 1992)].

Rhodamine-phallodin staining

Cells were fixed for 20 minutes in MBS + 3.7% formaldehyde. Samples were permeabilized with MBS + 0.1% Triton X-100 for 5 minutes, and then blocked in MBS + 0.1% Triton X-100 + 2% BSA for 10 minutes. Cells were stained for 20 minutes using 1 μg/ml rhodamine-phalloidin (Sigma) in blocking solution. All incubations were done at room temperature.

Cell spreading assay

Cells dissociated in CMFM (as described above) were plated into fibronectin- or poly-L-lysine-coated chambered coverslips (VWR). Fibronectin substrates containing a 'high' concentration of fibronectin were prepared: coverslips were coated with 0.2 mg/ml fibronectin (Sigma, diluted with MBS) for 3 hours at room temperature, and then

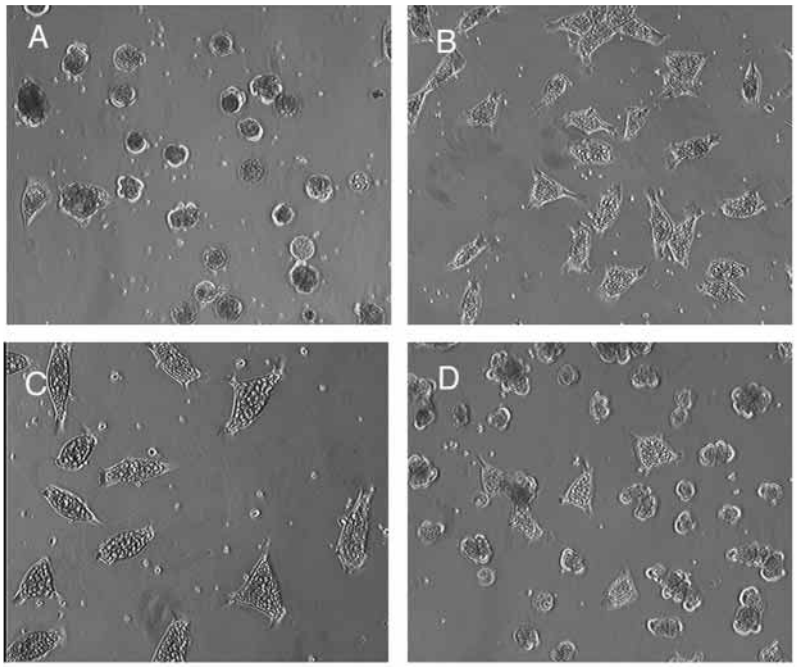


Fig. 1. Typical morphology of dissociated cells plated on fibronectin and analyzed in cell migration assays. (A) Uninjected, untreated animal cap. (B) Uninjected, activin-treated animal cap. (C) Head mesoderm, dissected from stage 10 embryo. (D) Dorsal marginal zone (chordamesoderm), dissected from stage 10 embryo.

blocked with BSA (50 mg/ml in MBS) for 30 minutes at room temperature. Poly-L-lysine-coated coverslips were prepared: coverslips were coated with a solution of 1 mg/ml poly-L-lysine (Sigma) in water for 30 minutes at room temperature. The coverslips were then rinsed 10 times with MilliQ H_2O, and finally blocked with BSA (50 mg/ml in MBS) for 30 minutes at room temperature.

For the dose response experiment, coverslips were coated as described here with the following concentrations of fibronectin: low (0.1 mg/ml), medium (0.15 mg/ml) and high (0.2 mg/ml).

After plating, cells were allowed to recover and spread on the substrate for 2 hours, at which time they were fixed and processed for rhodamine-phalloidin staining. A cell was scored as spread if it took on a spread morphology: flattened with irregular borders and exhibiting actin-rich protrusions extending along the substrate.

Mesendoderm extension assay

The mesendoderm extension assay was performed as described (Davidson et al., 2002). Coverslips were coated with 0.1 mg/ml fibronectin (Sigma, diluted with MBS) overnight at 4°C. Coverslips and plastic petri dishes were blocked with Danilchik's For Amy [DFA, containing 1 mg/ml BSA, as described previously (Davidson et al., 2002)] for at least 1 hour before explants were mounted. Timelapse analysis was performed using a Hamamatsu C2400 CCD Camera attached to a Zeiss Stemi SV11 Stereoscope. Images were captured and assembled into a timelapse file using Openlab software (Improvision).

RESULTS

Characteristics of the migration activity of activin-treated cells

To assay cell migration, we dissociated animal cap cells, treated them with activin protein, plated them onto fibronectin-coated coverslips and observed them by time lapse microscopy. Fibronectin is the natural and required component of the extracellular matrix upon which prechordal mesoderm cells crawl (Winklbauer and Keller, 1996). Animal cap cells not exposed to activin assume a characteristic round shape and remain stationary, although many exhibit circus movement, the rapid circling of an actin-rich protrusion around the cell (Fig. 1A) (Johnson, 1976). Activin-treated animal cap cells attach to the fibronectin substratum; they possess two flat bipolar lamellae similar to dissociated prechordal mesoderm cells (Fig. 1B,C). Prechordal mesoderm cells crawl at an average speed of 2.5 μm/minute (Winklbauer, 1990), comparable with activin-treated animal cap cells (Wacker et al., 1998). As the cells are plated onto a simple (uniform) fibronectin substrate, this assay does not measure directional cell migration; a cell is scored positive for migration if it exhibits both active protrusions and the ability to translocate upon the fibronectin substrate. A cell is scored negative for migration if it lacks the ability to translocate across the substrate, whether or not it has active protrustions.

Convergent extension, the other major motile activity in early mesoderm, was assayed in the intact animal cap by examining its asymmetric extension along one dimension. In our standard assay, animal caps are treated with activin protein for 1 hour and allowed to heal and develop until stage 19, when convergent extension-like movements are first easily recognizable. In experiments described here, cell migration and convergent extension were assayed in parallel, although it should be pointed out that these assays are not contemporaneous: cell migration can be assayed within 3 hours of plating, while convergent extension is not noticeable for ~18 hours. It should be noted that concentrations of activin used here are comparable with amounts used in many previous studies, in which markers of prechordal and chordamesoderm were induced in each respective assay (Green and Smith, 1990; Green et al., 1992; Symes et al., 1994; Gurdon et al., 1997; Gurdon et al., 1999).

VegT is necessary for both activin-induced cell migration and convergent extension

VegT, also known as *Xombi*, *Brat* and *Antipodean*, is a member of the T-box family of transcription factors, and is a direct

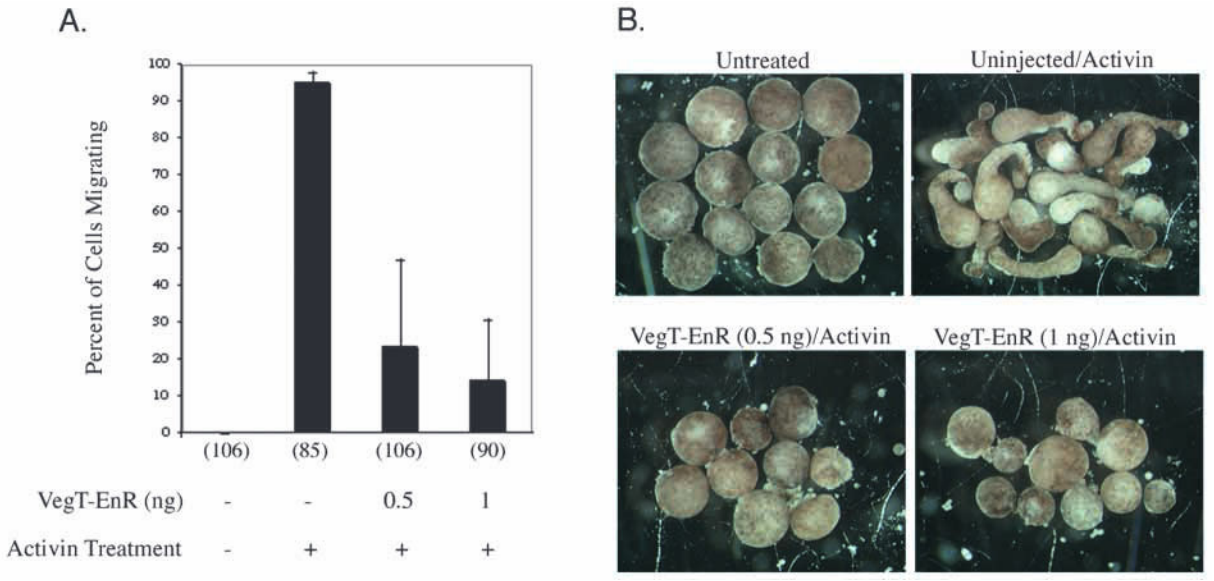


Fig. 2. *VegT* is necessary for activin-induced cell migration and convergent extension. Increasing amounts of *VegT*-EnR RNA (0.5-1 ng) were injected into the animal pole. Sibling animal caps were dissected at stage 9 and processed for each of the assays. (A) Cell migration. Numbers immediately below the graph in parentheses reflect total number of cells scored. (B) Convergent extension.

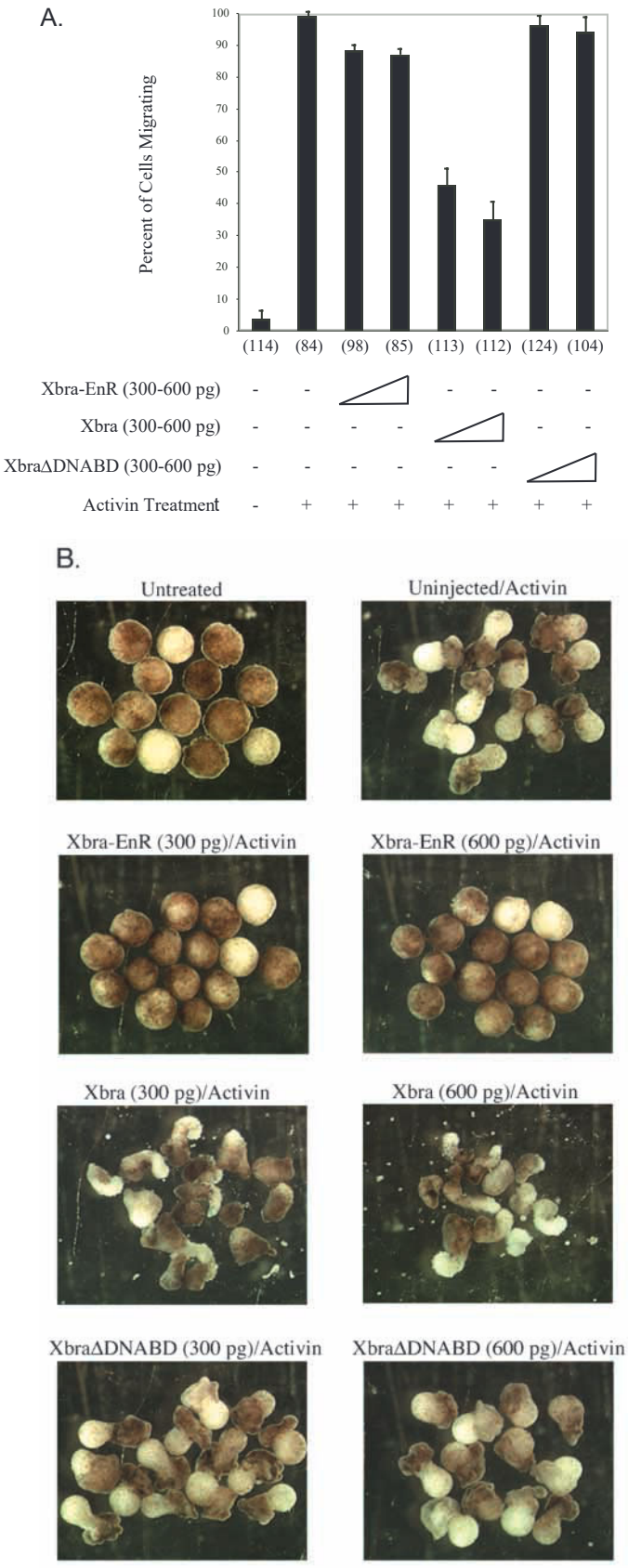
downstream target of activin signaling (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Ectopic expression of *VegT* can induce both endoderm and mesoderm. In addition, oocyte depletion (Zhang et al., 1998) shows that maternal *VegT* RNA is required for both endoderm and mesoderm development. We tested whether *VegT* is required for cell migration and convergent extension. A dominant inhibitory form of *VegT* (*VegT*-EnR) was constructed by fusing its DNA-binding domain to the repressor domain of the *Drosophila engrailed* gene; this form of *VegT* will repress its normal downstream targets. Previously, *VegT*-EnR has been demonstrated to inhibit expression of mesodermal markers (*Gsc*, *Xbra* and *Xlim-1*) and mesodermal patterning; *VegT*-EnR also inhibited blastopore formation (Horb and Thomsen, 1997). As might be expected, expression of *VegT*-EnR blocks both cell migration and convergent extension (Fig. 2). Cells injected with an inhibitory dose of *VegT*-EnR look like untreated animal cap cells. These data suggest that *VegT* activity is necessary for both activin-induced cell migration and convergent extension.

Xbra mutants reveal separable pathways for cell migration and convergent extension

Xenopus brachyury (*Xbra*), a second T-box transcription factor, is also induced by activin. Like *VegT*, it functions as a transcriptional activator (Kispert et al., 1995). *Xbra* is expressed throughout the marginal zone beginning at stage 9 and persists through gastrulation in the circumblastoporal ring, including the chordamesoderm undergoing convergent extension. It is also required for convergent extension (Conlon and Smith, 1999). However, *Xbra* expression is excluded from the prechordal mesoderm, and presumably is not required for prechordal mesoderm migration. As the specific exclusion of *Xbra* in that region could itself be important, three different forms of *Xbra* were tested in the cell migration and convergent extension assays: wild type, an inhibitory form (*Xbra*-EnR) and a DNA-binding domain mutant that is inactive (*Xbra*ΔDNABD) (Kispert and Hermann, 1993). As previously demonstrated, *Xbra*-EnR completely blocks convergent extension, but has minimal effects on cell migration (Fig. 3). By contrast, wild-type *Xbra* does not inhibit convergent extension, but partially inhibits cell migration in a dose-dependent manner. Convergent extension movements are not inhibited in caps injected with wild-type *Xbra*, although the caps look qualitatively different, thinner, than uninjected caps. *Xbra*ΔDNABD has virtually no effects on either cell migration or convergent extension, indicating that these phenotypes are mediated by downstream transcriptional targets of *Xbra*. Although *Xbra* functions to promote convergent extension, these data suggest that the specific exclusion of *Xbra* from the prechordal mesoderm could be important in allowing cell migration. This is consistent with the idea that *Xbra* is specifically repressed in cells undergoing cell migration in response to activin (Gurdon et al., 1999; Symes et al., 1994). The regulation of *Xbra* expression may therefore determine whether gastrula stage mesodermal cells undergo cell migration or convergent extension. This is in contrast to *VegT*, which is required for both cell migration and convergent extension.

The inhibition of cell migration by *Xbra* can be rescued by blocking convergent extension

To determine whether *Xbra* inhibits cell migration by promoting convergent extension, we blocked convergent extension with a secreted Wnt inhibitor. Specifically, it has been reported that Wnt11 is a direct transcriptional target of *Xbra*, and is necessary for convergent extension (Tada and Smith, 2000). As *Xbra*-EnR inhibits Wnt11 expression both in activin-treated animal caps, as well as in the endogenous mesoderm (Tada and Smith, 2000), we constructed a dominant-negative Wnt11 (dn Wnt11) that lacks the C-terminal 70 amino acid cysteine-rich domain (Tada and Smith, 2000). As shown



in Fig. 4, *Xbra* inhibits cell migration in a dose-dependent manner. However, co-expression of dn Wnt11 partially rescues cell migration. These data suggest that blocking convergent extension impairs the ability of *Xbra* to inhibit cell migration. Therefore, we propose that *Xbra* inhibits cell migration by actively promoting convergent extension. In this view, cell migration is a default state of activin stimulation, and *Xbra* acts as a switch between cell migration and convergent extension.

Wnt11 is required for convergent extension, but not cell migration

Because dominant-negative Wnt11 partially restores cell migration inhibited by *Xbra*, it is possible that the Wnt pathway regulates both convergent extension and cell migration. Therefore, a role for Wnt11 in cell migration was tested. As demonstrated previously, dn Wnt11 partially blocks convergent extension (Fig. 5B) (Tada and Smith, 2000); however, cell migration is unaffected (Fig. 5A). This partial inhibition correlates with the partial effects seen in counteracting the *Xbra*-mediated inhibition of cell migration.

Levels of Wnt11 signaling seem to be crucial for convergent extension, as reported previously (Djiane et al., 2000). It has been shown that both overexpression and inhibition of Wnt11 signal inhibit convergent extension. As demonstrated previously, overexpression of Wnt11 blocks convergent extension (Fig. 5B) (Tada and Smith, 2000); however, cell migration is unaffected (Fig. 5A). Because Wnt11 is not sufficient to inhibit cell migration, it cannot solely account for the inhibition of cell migration by *Xbra*. Wnt11 may act in conjunction with other factors in order to block cell migration and promote convergent extension.

Xbra inhibits cell shape changes induced by dominant active Rac or dominant active Cdc42

As the control of cell migration is largely unexplored in embryos, the downstream targets through which *Xbra* inhibits cell migration remain largely unknown. Downstream transcriptional targets could act on any number of processes in order to inhibit cell migration, for example, extracellular signaling, intracellular signaling to the cytoskeleton, actin polymerization or cellular adhesion. To define better the mechanisms by which *Xbra* inhibits cell migration, we examined the response of the cells to small GTPases involved in the cytoskeleton and adhesion.

The Rho family of small GTPases has been shown in cultured cell lines to have specific effects on cell morphology and the actin cytoskeleton. Activated Cdc42 injected into cells induces filopodia, while activated Rac induces lamellipodia (Nobes and Hall, 1995). *Xenopus* animal cap cells respond similarly to these activated GTPases. To avoid early embryonic phenotypes, V12 Cdc42 or V12 Rac were injected as expression plasmids into both blastomeres at the two-cell stage, thereby limiting expression to post mid-blastula

Fig. 3. *Xbra* mutants reveal separable pathways for convergent extension and cell migration. Embryos were injected with 300 or 600 pg of RNA encoding either *Xbra*-EnR, wild type *Xbra* or *Xbra*ΔDNABD. Sibling animal caps were dissected at stage 9 and processed for each of the assays. (A) Cell migration. Numbers immediately below the graph in parentheses reflect total number of cells scored. (B) Convergent extension.

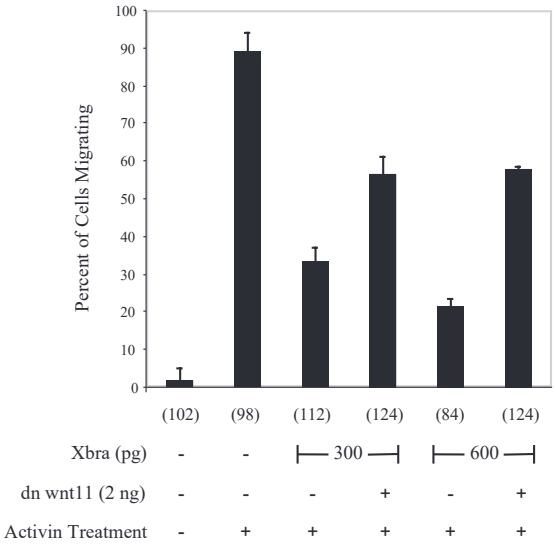


Fig. 4. The *Xbra*-mediated inhibition of cell migration can be partially rescued by an inhibitor of convergent extension. Embryos were injected with 300 or 600 pg of RNA encoding wild-type *Xbra*, plus or minus dominant-negative Wnt11 (2 ng RNA). Animal caps were dissected at stage 9 and processed for the cell migration assay. Numbers immediately below the graph in parentheses reflect total number of cells scored.

transition. Under these conditions, V12 Cdc42 induces filopodia and V12 Rac induces elaborate lamellipodia (Fig. 6A). When wild-type *Xbra* is co-injected with the activated form of either GTPase, the cell shape changes are inhibited (Fig. 6B); the cells have the same morphology as those that have not been injected. By contrast, co-injection of *Goosecoid* (*Gsc*), a transcription factor expressed in the prospective prechordal mesoderm, has negligible effects (data not shown).

Because *Xbra* can inhibit cell shape changes induced by activated Cdc42 or Rac, this suggests that the effect of *Xbra* is not restricted to the signaling pathways upstream of the GTPases. More likely, its effect is on the signaling pathways downstream of Cdc42 and Rac that lead to regulated actin polymerization and cell morphology changes. However, this assay cannot distinguish between inhibition of actin polymerization and inhibition of cell adhesion to the fibronectin substrate.

Xbra inhibits cell spreading on fibronectin, but not poly-L-lysine

To distinguish between these two possibilities, cell spreading assays were performed. When a twofold higher concentration of fibronectin is used to coat coverglasses (see Materials and Methods), dissociated, uninjected animal cap cells (not treated with activin) spread upon the substrate. The cells are flattened, exhibiting an irregular border and few small spiky protrusions (Fig. 6C). By comparison, cells plated on a poly-L-lysine substrate also spread, exhibiting an irregular border and, often, large flat lamellae (Fig. 6C). Because the injection of activated GTPases is not necessary for the cells to exhibit these morphological behaviors, this represents a simple assay for adhesion and spreading upon a substrate. Injection of *Xbra* inhibits cell spreading upon fibronectin, such that the cells assume a round, unspread, unadherent morphology (Fig. 6C,D). However, expression of *Xbra* has no effect on spreading upon poly-L-lysine. Fig. 6E shows dose response to *Xbra* in the cell spreading assay upon fibronectin. Three fibronectin concentrations were tested: low, medium and high. Low is

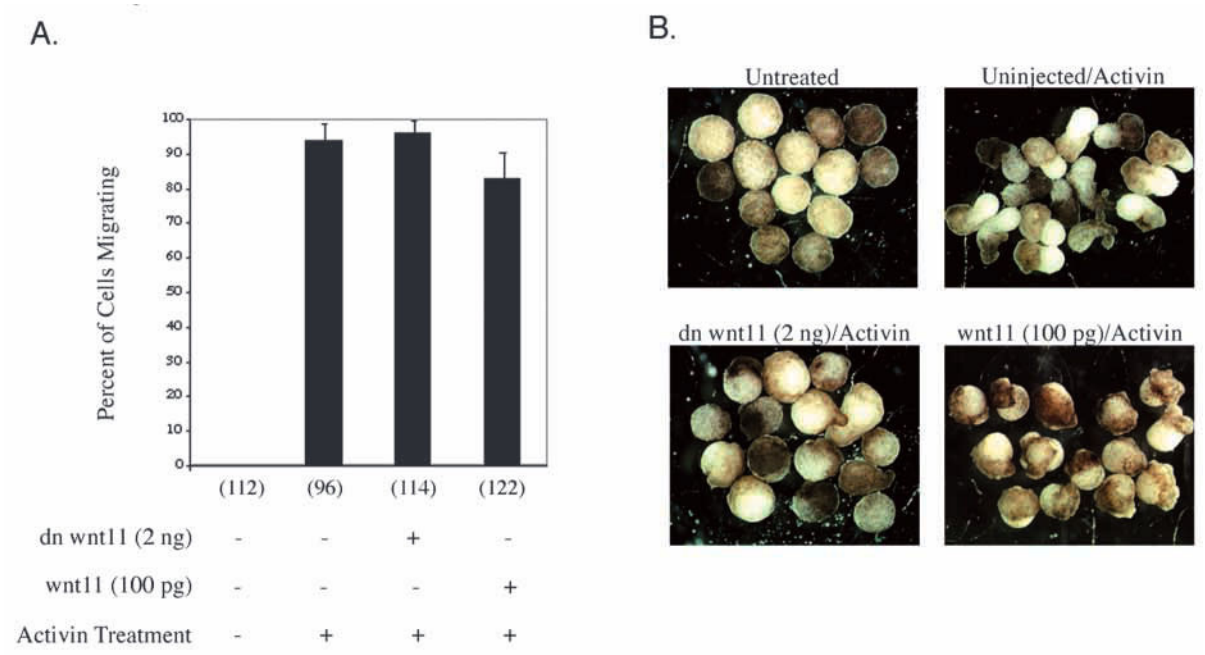


Fig. 5. Wnt11 is a downstream target of *Xbra* that is necessary for convergent extension but not for cell migration. Levels of Wnt11 signal dramatically affect convergent extension, but similarly have no effect on cell migration. Embryos were injected with either dominant-negative Wnt11 (2 ng RNA) or wild-type Wnt11 (100 pg). Sibling animal caps were dissected at stage 9 and processed for each of the assays. (A) Cell migration. Numbers immediately below the graph in parentheses reflect total number of cells scored. (B) Convergent extension.

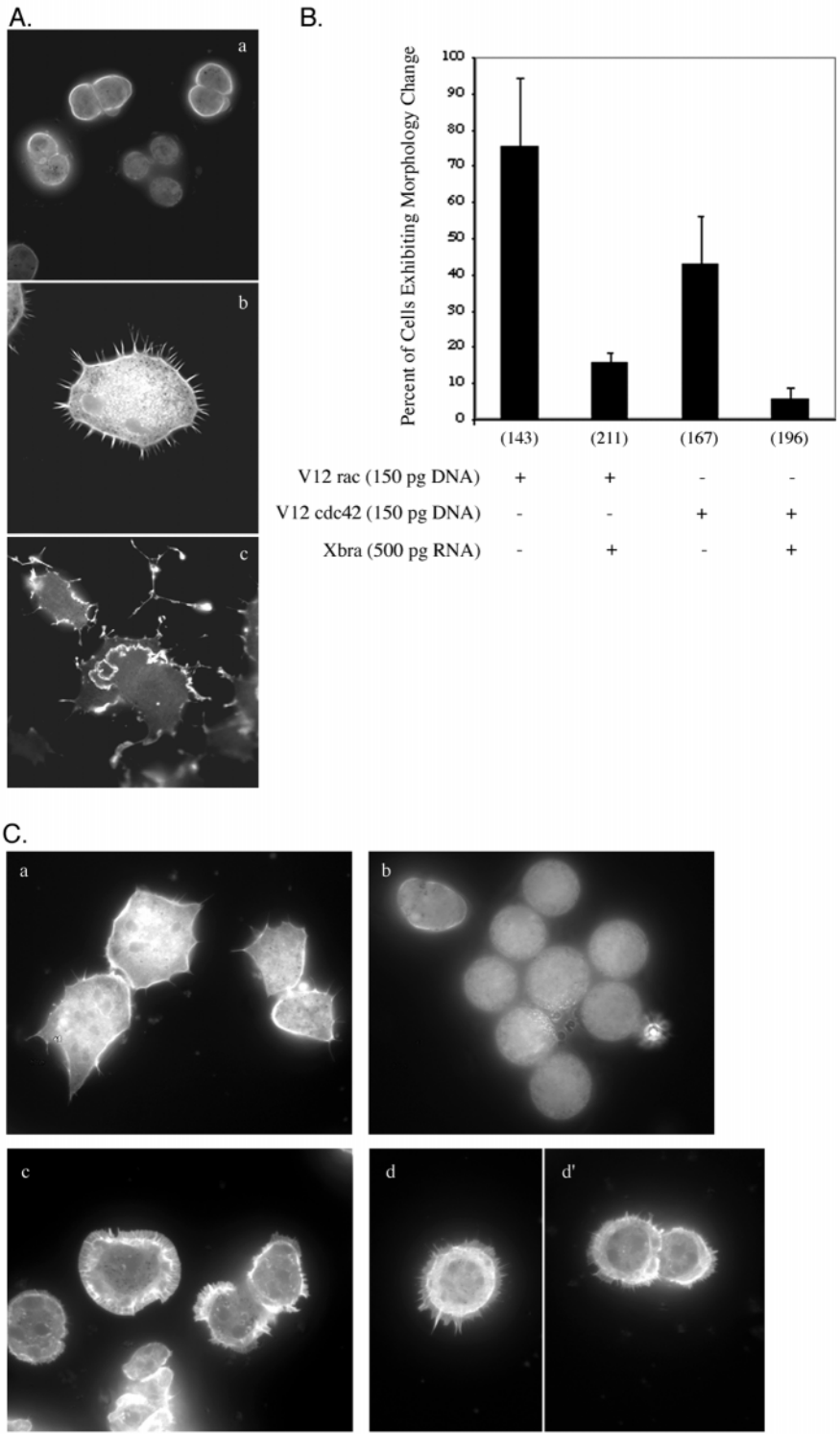
equal to the amount used in previous migration assays, and high is equal to the amount used in Fig. 6D for the previous cell spreading assay (see Materials and Methods for details). These data suggest that in a simple assay to test adhesion and cell spreading, *Xbra* can inhibit adhesion specifically to fibronectin. Thus, the effects mediated by *Xbra* are less likely to be general effects upon the cytoskeleton and contractility, and may be specific to the process of adhesion to fibronectin.

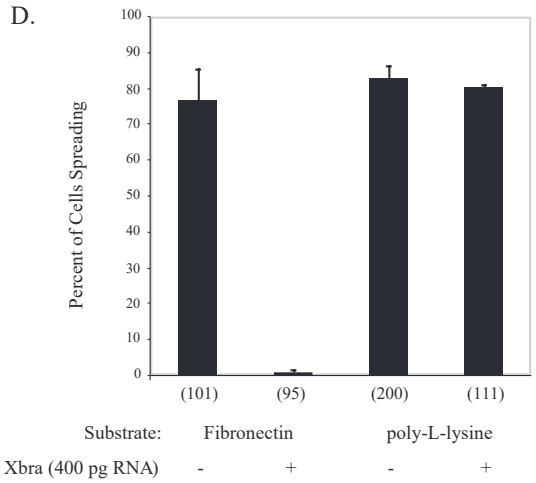
It is worth noting that *Xbra*-mediated inhibition of cell spreading in this assay is much more potent than its inhibition of activin-induced cell migration (compare Fig. 3A with Fig. 6D,E). Comparable amounts of *Xbra* inhibit activin-induced cell migration by 50-60%, but inhibit cell spreading on fibronectin almost completely. This difference is likely due to the effect of activin treatment. It has been demonstrated that activin, in inducing cell migration, upregulates adhesion to fibronectin (Wacker et al., 1998). This upregulation of adhesion probably accounts for the relative lower potency of *Xbra* in the migration assay, and higher potency in the cell spreading assay, in which cells are not treated with activin.

In addition to cell spreading assays, visual actin polymerization assays were performed to test the ability of small GTPases to induce actin polymerization in extracts (Ma et al., 1998). Small scale extracts were made from animal caps from either uninjected embryos or embryos injected animally with *Xbra* (500 pg RNA) at the two-cell stage. GTPγS-loaded Cdc42 and GTPγS-loaded Rac induced actin polymerization equally well in extract from uninjected animal caps as in extract from *Xbra*-injected animal caps (S. Eden, K.M.K. and M.W.K., unpublished). Taken together, these data suggest that *Xbra* inhibits adhesion, not actin polymerization, and, more specifically, adhesion of cells to a fibronectin substrate.

The effects of *Xbra* are recapitulated in the endogenous populations of cells that undergo either cell migration or convergent extension, in the marginal zone

We have used activin-induced animal caps to recreate, as best as possible, conditions in the marginal zone, where endogenous populations of cells undergo either migration or convergent extension. We therefore wished to test whether *Xbra* has the same effects in the marginal zone cells themselves. For this, we injected *Xbra* into the marginal zone of the two dorsal cells of a four-cell embryo. At stage 10, head mesoderm was dissected from the resulting gastrulae, and the dissociated cells assayed. As shown in Fig. 7A, expression of *Xbra* inhibits the ability of the prechordal mesoderm cells to migrate, in a dose-dependent manner. Sibling embryos were cultured until early tailbud stages, and, as shown, overexpression of *Xbra* in the dorsal marginal zone causes anterior truncations (Fig. 7B). Therefore, even though migration is inhibited in only 50% of prechordal mesoderm cells, this is clearly enough to cause severe defects in head





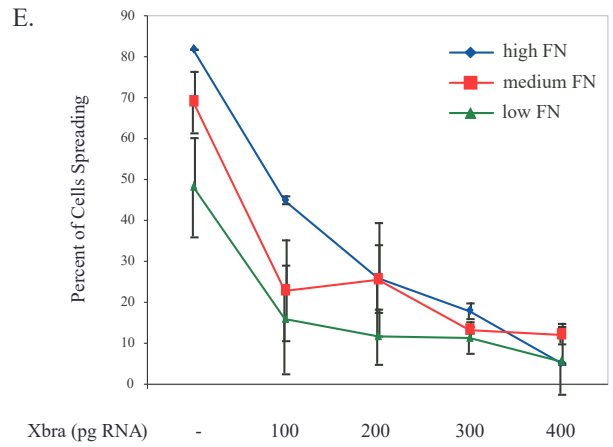


Fig. 6. *Xbra* inhibits cell shape changes induced by V12 Rac or V12 Cdc42, and cell spreading on fibronectin but not poly-L-lysine. (A) V12 Rac and V12 Cdc42 induce cell shape changes similar to those exhibited by cultured cells. Embryos were injected with 150 pg DNA of either V12 Cdc42 or V12 Rac into each blastomere. Animal caps were dissected at stage 10, dissociated and plated onto a fibronectin-coated coverslip. Cells were then stained for actin filaments using rhodamine-labeled phalloidin. (a) Uninjected animal cap cells. (b) Animal cap cell expressing V12 Cdc42. (c) Animal cap cell expressing V12 Rac. (B) *Xbra* inhibits cell shape changes induced by the activated GTPases. *Xbra* (500 pg RNA) was co-injected with either V12 Rac or V12 Cdc42 (150 pg DNA). Plated animal cap cells were fixed and the number of cells exhibiting a morphology change such as those in Fig. 5A were counted. Numbers immediately below the graph in parentheses reflect total number of cells scored. When normalized for number of cells which respond to V12 Rac or V12 Cdc42, percent inhibition is as follows: for V12 Rac, 77.92% (±6.37%), for V12 Cdc42, 86.06% (±6.11%). (C) Uninjected, dissociated animal cap cells spread upon fibronectin and poly-L-lysine substrates. (a) Uninjected cells plated onto fibronectin. (b) *Xbra*-injected cells (400 pg RNA) plated onto fibronectin. (c) Uninjected cells plated onto poly-L-lysine. (d,d′) *Xbra*-injected cells (400 pg RNA) plated onto poly-L-lysine. (D) *Xbra* inhibits cell spreading on fibronectin but not poly-L-lysine. Uninjected or *Xbra*-injected (400 pg RNA) animal cap cells were plated onto either fibronectin or poly-L-lysine. Plated cells were fixed and stained with rhodamine-phalloidin to visualize actin-rich protrusions. Cells exhibiting a spread morphology, i.e. flattened with irregular borders and often projecting actin-rich protrusions along the substrate, were counted. Numbers immediately below the graph in parentheses reflect total number of cells scored. (E) Dose-response of the effects of *Xbra* on cell spreading on fibronectin. See text and Materials and Methods for details regarding fibronectin concentrations. At least 170 cells were scored for each data point.

development. Another assay was used to test prechordal mesoderm migration. Davidson et al. have developed an assay to test the rate and extent of mesendoderm extension, in which the head mesoderm migrates upon a fibronectin substrate as an intact mantle (Davidson et al., 2002). Timelapse analysis was performed to compare mesendoderm extension in dorsal marginal zone explants (see Materials and Methods) from *Xbra*-injected, uninjected and *Xbra*-EnR-injected embryos (explants numbered 1, 2, and 3, respectively in Fig. 7C). As shown, the *Xbra*-injected explant initially extends at a similar rate as the other explants. However, by about 7.5 hours (Fig. 7C), the *Xbra*-injected explant has reached its maximum extension, while the uninjected and *Xbra*-EnR-injected explants continue to extend for another 2.5 hours (Fig. 7C). Thus, even as an intact mantle, inhibition of migration by 50% causes a clear decrease in the extent of mesendoderm migration. Meanwhile, migration of the *Xbra*-EnR-injected explant was indistinguishable from that of the uninjected explant, not surprisingly, as expression of *Xbra*-EnR has no effect in the in vitro cell migration assay.

In a complemetary set of experiments, the effect of blocking *Xbra* function in the chordamesoderm was tested. *Xbra*-EnR was injected into the marginal zone of the two dorsal cells of a four-cell embryo, and the chordamesoderm region of the dorsal marginal zone was dissected from the resulting gastrulae. As reported previously, expression of *Xbra*-EnR in the dorsal marginal zone inhibits its ability to undergo convergent extension (Fig. 8A). Embryos cultured to early tailbud stages exhibit a phenotype indicative of inhibition of convergent extension: short trunk and tail with a fully formed head (Fig. 8B).

We then asked whether inhibition of *Xbra* activity (by expression of *Xbra*-EnR) promoted cell migration. Cells from the dorsal marginal zone, which undergo convergent extension, do not migrate processively, although a fraction of them spread on a fibronectin substrate (Fig. 1D) (Wacker et al., 1998). However, expression of *Xbra*-EnR in these cells causes an increased fraction of them to undergo cell spreading and migration (Fig. 8C). This effect can be rescued with equal amounts of wild type *Xbra* RNA. Therefore, in this context, blocking *Xbra* function leads to increased adhesion to fibronectin and cell migration. This suggests that a normal function of *Xbra* expression in the marginal zone is to inhibit adhesion to fibronectin and, therefore, cell migration.

The effects of *Xbra* on the behavior of chordamesoderm cells can be recapitulated by mutants of *Xdsh* that inhibit the Wnt PCP pathway

We wondered to what extent the effects of *Xbra* inhibition in the marginal zone could be recapitulated by inhibition of the Wnt signaling pathway, specifically the planar cell polarity pathway which has been shown to be necessary for convergent

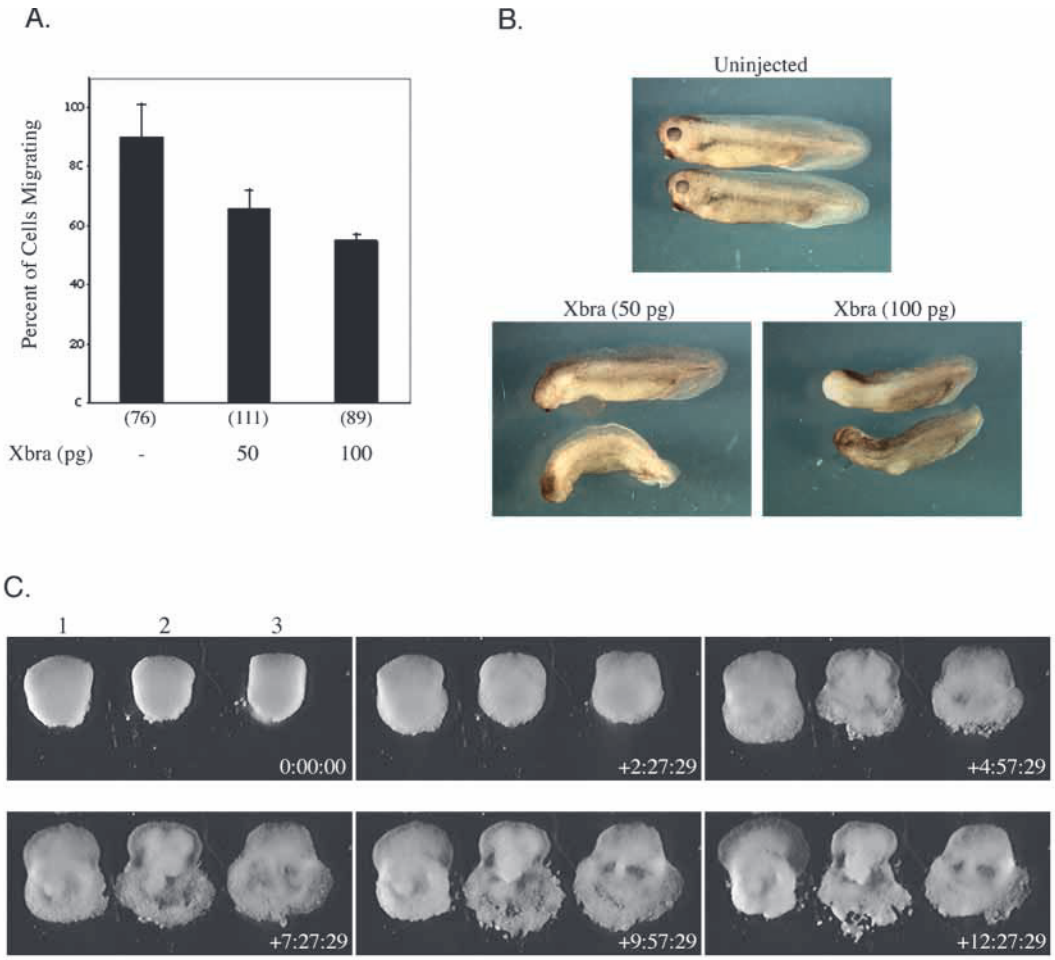


Fig. 7. *Xbra* has effects in the marginal zone consistent with an in vivo role for inhibiting cell migration and promoting convergent extension. Wild-type *Xbra* inhibits cell migration of the head mesoderm and gives rise to anteriorly truncated embryos. Amounts of RNA listed are per blastomere. (A) Head mesoderm migration. Wild-type *Xbra* was injected into the dorsal marginal zone (two cells from a four-cell embryo). Prechordal mesoderm was dissected out of resulting stage 10 embryos. Numbers immediately below the graph in parentheses reflect total number of cells scored. (B) Whole embryo phenotype. (C) Mesendoderm extension assay. Selected frames from a timelapse are shown, with time relative to the first frame shown in the bottom right corner. Explants are numbered 1-3 above the first frame. (1) Explant injected with *Xbra* (100 pg). (2) Uninjected explant. (3) Explant injected with *Xbra*-EnR (400 pg).

extension (Wallingford and Harland, 2001). To determine whether the effect of *Xbra* upon cell behavior can be largely attributed to downstream effects on cell fate or a more direct effect upon cell motility, different *Dishevelled* mutants were tested within the chordamesoderm for effects on cell migration. *Dishevelled* (*Dsh*) is an intracellular mediator of Wnt pathway signal transduction. It is a modular protein with multiple functions: the DIX domain mediates cell fate decisions via the Wg/Wnt pathway, while the PDZ domain mediates effects on cell behavior via the planar cell polarity (PCP) pathway (Rothbacher et al., 2000; Wallingford et al., 2000). We tested whether the effects of *Xbra*-EnR on chordamesoderm cell behavior could be recapitulated with mutants of *Dsh* that specifically block the planar cell polarity pathway downstream of a Wnt signal, in this case specifically Wnt11. *Xdsh*ΔDIX (deletion of the DIX domain), *Xdsh*ΔPDZ (deletion of the PDZ domain) and Xdd1 (deletion of most of the PDZ domain and part of the following region) were tested for effects on both convergent extension and cell migration within the chordamesoderm.

As reported previously, chordamesoderm explants expressing *Xdsh*ΔPDZ and Xdd1 fail to elongate; explants expressing *Xdsh*ΔDIX are also partially inhibited from undergoing convergent extension (Fig. 9A). As shown in previous figures, explants expressing either dn Wnt11 or *Xbra*-EnR are also inhibited in convergent extension movements. However, these same chordamesoderm explants, when assayed for cell migration, reveal that overexpression of specific *Xdsh* mutants (as well as dn Wnt11), causes an increased fraction of cells to undergo cell spreading and migration on a fibronectin substrate (Fig. 9B). It is notable that the mutants that inhibit the Wnt PCP pathway do so without altering cell fate in the dorsal mesoderm, as measured by staining for differentiated muscle and notochord (using the antibodies Tor70, MZ15 and 12/101), as well as Northern blot analysis of the genes *Xlim1*, *Xnr3*, *Gsc*, *Xotx2*, *Chordin* and *Xbra* (Sokol, 1996; Wallingford and Harland, 2001). These data suggest that specific inhibition of the Wnt PCP pathway, which does not alter cell fate, is sufficient to partially switch dorsal mesoderm cells from convergent extension behavior to cell migration. In addition,

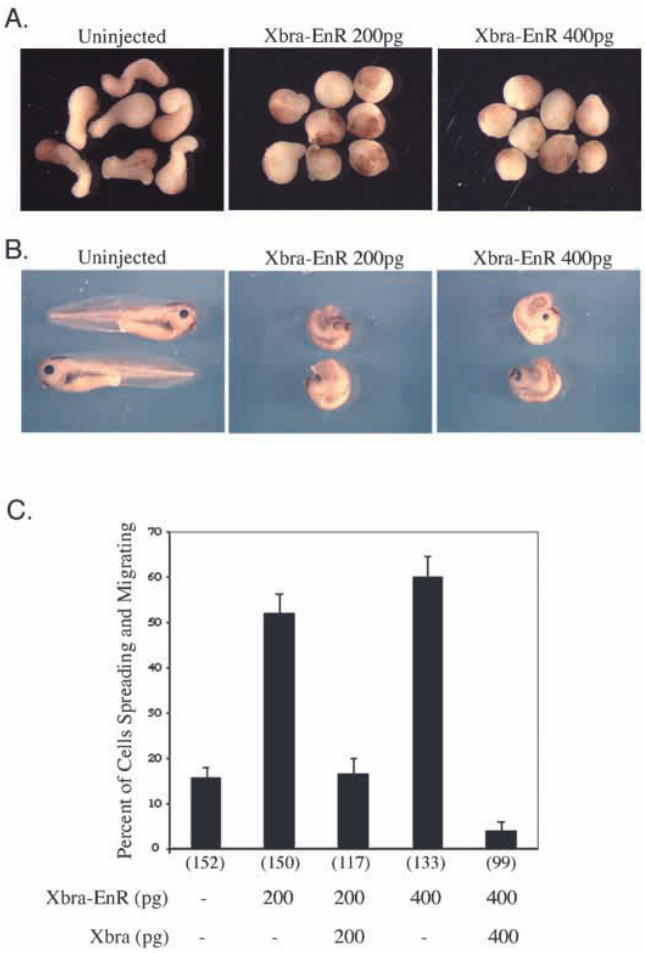


Fig. 8. *Xbra*-EnR inhibits convergent extension of the dorsal marginal zone, giving rise to tadpoles with fully formed heads, but very short trunks. In addition, inhibiting *Xbra* function in the dorsal marginal zone increases the number of cells which spread and crawl on fibronectin. (A) DMZ convergent extension. (B) Whole embryo phenotype. (C) DMZ cell migration. Numbers immediately below the graph in parentheses reflect total number of cells scored.

cell migration may be a default state within the dorsal mesoderm, which requires active signals to inhibit components of the migration machinery, such as adhesion to fibronectin, and promotes convergent extension.

DISCUSSION

During early development in *Xenopus* embryos, the regions destined to give rise to the head and trunk organizers are juxtaposed. Gene expression differences arise between these two regions: differences that not only define much later cell and tissue fates, but which also give rise to immediate differences in cell behavior during gastrulation. Cells in the anterior-most region migrate, spreading out over the blastocoel roof. Cells immediately behind them converge toward the midline, ultimately leading to extension of the body axis and forming notochord.

Xbra is a transcription factor that appears early in the trunk compartment of the organizer, where it has long been known to have a role in trunk mesoderm development. We demonstrate here that *Xbra* actively inhibits cell migration, both in activin-induced animal cap cells, and in the endogenous prechordal mesoderm. This inhibition of cell migration can be rescued by inhibiting convergent extension. These experiments suggest that in the embryo, *Xbra* acts as a morphogenetic switch not only promoting convergent extension, but also actively repressing cell migration. As expected for such a switch, blocking *Xbra* activity in the dorsal mesoderm via dominant-negative *Xbra* (*Xbra*-EnR) increases the number of cells in the chordamesoderm that spread on fibronectin and actively migrate. Under these conditions, the default behavior of the tissue appears to be cell migration. Therefore, one unappreciated role of endogenous *Xbra* is to inhibit cell migration within the chordamesoderm, while fostering convergent extension movements. *Xbra*, which has a role in the eventual cytodifferentiation of the chordamesoderm, also has an immediate dual role in morphogenetic movements.

Wnt11 is a direct downstream target of *Xbra* which is necessary for convergent extension (Tada and Smith, 2000). Although Wnt11 signaling is not required for cell migration (Fig. 5A), expression of dominant-negative Wnt11 reverses the inhibitory effects of *Xbra* on activin-induced cell migration. These same effects can be produced in the dorsal mesoderm, with dn Wnt11 as well as deletion mutants of *Dishevelled*, which inhibit the Wnt planar cell polarity pathway (*Dsh*ΔPDZ, Xdd1). Notably, these *Dsh* mutants have no effect on cell fate (Sokol, 1996; Wallingford and Harland, 2001).

Taken together, these data suggest that there exist within the dorsal mesoderm two cell behaviors, with cell migration as a default state. Expression of *Xbra* within the prospective chordamesoderm produces a domain where cell migration is inhibited. The ground state may be restored by inhibition of *Xbra* itself or the downstream Wnt planar cell polarity pathway. In the embryo, *Xbra* is excluded from the prechordal mesoderm, allowing these cells to exhibit active cell migration. *Xbra* acts as a cell behavior switch that is crucial for the proper anteroposterior development of the embryo. Inhibition of *Xbra* activity in the chordamesoderm results in embryos with a shortened trunk, a consequence of a failure of convergent extension; these cells now actively migrate. Misexpression of *Xbra* in the prechordal mesoderm results in embryos with head truncations, as a consequence of a failure of cell migration.

It has been reported that *Gsc* and *Mix.1*, two transcription factors expressed in the dorsal marginal zone of gastrula stage embryos, synergize to repress the expression of *Xbra* (Latinkic and Smith, 1999). Expression of dominant inhibitory forms of either transcription factor results in inappropriate expression of *Xbra* in the prechordal mesoderm, and anterior truncation of the embryos. From the results presented here, it is likely in those experiments that derepression of *Xbra* in the prechordal mesoderm inhibited prechordal mesoderm migration, producing anterior truncations.

Niehrs and colleagues have reported on the gene anti-dorsalizing morphogenetic protein (ADMP), expressed in the chordamesoderm, which also seems to be part of a network that maintains the subdivision between prechordal and chordamesoderm (Dosch and Niehrs, 2000). It will be interesting to explore the relationship between ADMP and *Xbra*. It has already been shown that ADMP can induce the expression of *Xbra* (Moos et al., 1995), but whether *Xbra* also affects the

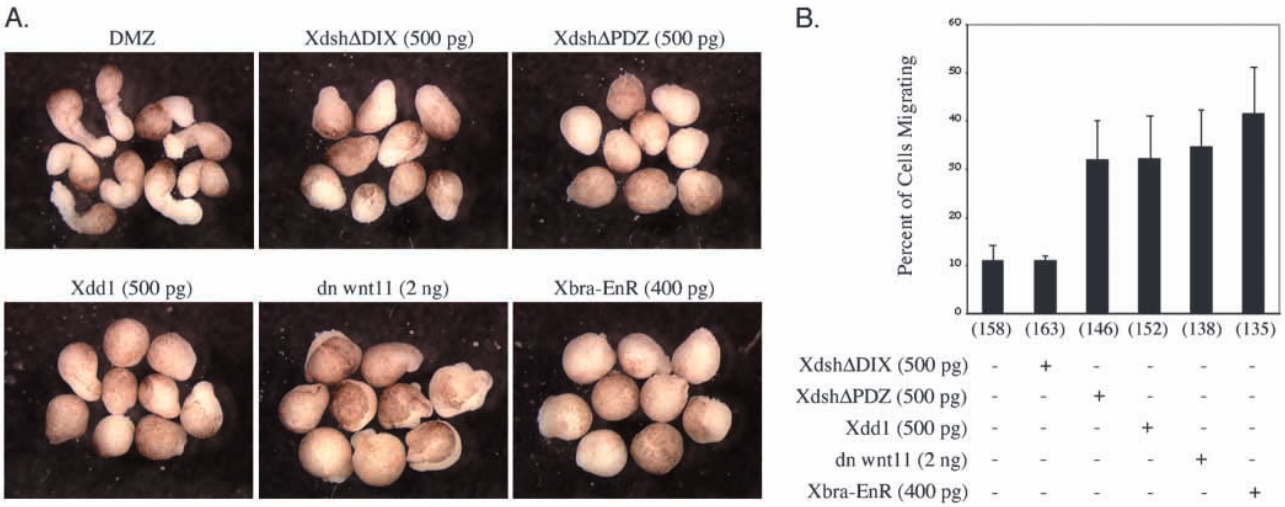


Fig. 9. *Xdsh* mutants that block the PCP pathway inhibit convergent extension and promote cell migration. (A) DMZ convergent extension. (B) DMZ cell migration. Numbers immediately below the graph in parentheses reflect total number of cells scored.

expression of ADMP is unclear. Similarly, the effects of ADMP specifically on cell behavior have yet to be determined. Although ADMP is expressed solely in the chordamesoderm, *Xbra* is expressed throughout the mesoderm in a circumblastoporal ring and is specifically excluded from the prechordal mesoderm. *Xbra* may be expressed beyond the chordamesoderm to control various degrees of convergent extension required throughout the mesoderm to ensure proper blastopore closure via radiolateral convergent extension movements.

The fact that *Xbra* expression is maintained in chordamesoderm but not paraxial mesoderm may be important for establishing an axis and polarity for mediolateral convergent extension. In terms of inducing convergent extension in paraxial mesoderm, prolonged *Xbra* expression may not be required. There may be other signals, specifically from the chordamesoderm, that propagate mediolateral convergent extension behavior, and there may be other genes induced within the paraxial mesoderm itself that play a role in the behavior of its cells.

Just how does *Xbra* inhibit cell migration? Ectopic expression of *Xbra* has several effects: inhibition of cell migration, inhibition of the morphological effects elicited by activated Rac and Cdc42 in dissociated animal cap cells, and inhibition of adhesion to fibronectin in a simple cell spreading assay. The inhibition of cell migration depends on the ability of *Xbra* to bind DNA and activate transcription, which suggests that these effects are mediated by one or more downstream transcriptional targets of *Xbra*. Wnt11, although required for convergent extension, is not sufficient to inhibit cell migration. Therefore, *Xbra* must induce the expression of other factors which can, at the very least, inhibit adhesion to fibronectin, either alone or in conjunction with Wnt11. It will be interesting to learn the downstream target genes of *Xbra* that may be involved in cell adhesion and movement.

In both the activin-induced animal cap system and the endogenous chordamesoderm, expression of *Xbra* or *Xbra*-EnR alters expression level of many markers as early as stage 10, including *Xbra* itself, *Mix.1*, *Xwnt-8*, *Gsc*, *Pintallavis*, *Xnot*, *Chordin*, *Noggin* and Wnt11 (Conlon and Smith, 1999; Tada and Smith, 2000). Although changes in any of these genes may represent a change in cell fate, it is notable that some of the effects described here can be recapitulated using mutants of *Dsh* that do not alter cell fate in the dorsal mesoderm.

In summary, vertebrate embryos possess two distinct cell behaviors that pattern the dorsal side of the embryo. Convergent extension, which can be assayed only in populations of cells, leads to notochord formation and patterns the trunk. Cell migration, which can be studied in individual cells, leads to different cell fates and inductive effects, which are confined to the anterior neural plate. Distinct as they are, in *Xenopus*, these two behaviors arise from adjacent cell populations; both can be produced downstream of the same signaling protein, activin. The distinction in cell behavior depends on the domain of *brachyury* expression. *Brachyury* actively inhibits cell migration while inducing the tissue to undergo convergent extension via the Wnt planar cell polarity pathway. However, Wnt11 is not sufficient to inhibit cell migration. Therefore, the downstream targets of *Xbra* that inhibit cell migration are unknown, but are capable of inhibiting cell shape changes induced by small GTPases and adhesion to fibronectin in a simple cell spreading assay. These findings raise two further questions: what is the mechanism by which *Xbra* regulates convergent extension and cell migration, and what establishes the domains of cell behavior? The key to both of these is the regulation of *Xbra* expression and its downstream targets.

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