Development 129, 2823-2834 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5021

A novel *Xenopus* Smad-interacting forkhead transcription factor (XFast-3) cooperates with XFast-1 in regulating gastrulation movements

Michael Howell, Gareth J. Inman and Caroline S. Hill*

Laboratory of Developmental Signalling, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
*Author for correspondence (e-mail: caroline.hill@cancer.org.uk)

Accepted 28 March 2002

SUMMARY

In early *Xenopus* embryos, the prototypical XFast-1/Smad2/Smad4 complex ARF1 is induced at the *Mix.2* ARE by activin overexpression. We have characterised ARF2, a related, but much more abundant, complex formed during gastrulation in response to endogenous TGFβ family members and we have identified a novel Fast family member, XFast-3, as its transcription factor component. Endogenous ARF2 efficiently competes out ARF1 at early gastrulation, due to the ability of XFast-3 to interact with activated Smads with much higher affinity than XFast-1. We demonstrate that ARF1 and ARF2 are activated by distinct TGFβ family members. Using morpholino antisense oligonucleotides to deplete levels of the constituent transcription factors XFast-1 and XFast-3 specifically, we demonstrate an important role for ARF1 and ARF2 in early *Xenopus* embryos in controlling the convergent extension movements of gastrulation.

Key words: Fast, Forkhead/winged helix, Gastrulation, Smad, TGFβ signalling, *Xenopus*

INTRODUCTION

The establishment of the three germ layers of the vertebrate embryo requires both inductive events and co-ordinated cell movements. In the *Xenopus* embryo, mesoderm is induced in the marginal zone by vegetally produced signals (Nieuwkoop, 1969). During gastrulation, the mesoderm involutes, and the dorsal mesoderm together with the overlying ectoderm converge and extend to close the blastopore and elongate the body axis (Keller, 1991). TGFβ superfamily members functionally related to activin (such as Xnr1, 2, 4, 5 and 6 and derrière) play crucial roles in both the specification of mesoendodermal cell fates and in gastrulation movements (reviewed by Schier and Shen, 2000; Smith, 1995). An analogous role for nodal family members in mice and zebrafish is also established (Beddington and Robertson, 1999; Feldman et al., 1998). However the molecular mechanisms by which these ligands regulate these processes are still very poorly understood.

The activin signal transduction pathway is well characterised, and the pathways activated by the related ligands are thought to be similar. Receptor activation leads to phosphorylation and activation of receptor-regulated Smads (Hill, 2001). In early *Xenopus* embryos, this is primarily XSmad2 (Faure et al., 2000), as there is little or no XSmad3 present at this time (Howell et al., 2001). Activated XSmad2 forms a complex with a co-Smad, which in the blastula and early gastrula is XSmad4β (Howell et al., 1999). The XSmad2/XSmad4β complexes accumulate in the nucleus and are recruited to distinct promoter elements by site-specific transcription factors. The paired-like homeodomain proteins Mixer, Milk and Bix3 can recruit active Smad complexes to the distal element (DE) of the *goosecoid* promoter (Germain et al., 2000; Randall et al., 2002), whereas the forkhead/winged-helix transcription factor XFast-1 recruits active Smad complexes to the activin responsive elements (AREs) in the *Mix.2* promoter and *Xnr1* enhancer (Chen et al., 1996; Osada et al., 2000). Although the DNA binding specificity of these transcription factors is very different, their interaction with the activated Smad complex occurs in all cases through a common short Smad interaction motif (SIM) (Germain et al., 2000).

Two distinct XSmad2/XSmad4β-containing complexes bind the *Mix.2* ARE (Howell et al., 1999). One, ARF1 (formally ARF for activin responsive factor) (Chen et al., 1996) is activated in *Xenopus* embryos by overexpressing activin. It comprises XFast-1, XSmad2 and XSmad4β, and is detected in nuclear extracts at approximately 80 minutes post stage 8 (Chen et al., 1996; Howell et al., 1999). The other is a distinct complex that we have named ARF2, and is distinguished from ARF1 by its faster mobility on a bandshift gel, its inducibility and time of appearance. It contains XSmad2 and XSmad4β, is activated by endogenous signalling molecules and is readily detected in nuclear extracts from uninjected embryos at about 240 minutes post stage 8 (Howell et al., 1999). ARF2 is the only ARF detected in uninjected embryos and perhaps, therefore, the most quantitatively important.

We have identified a novel Fast family member, XFast-3, which is expressed only during gastrulation, and is the

transcription factor component of ARF2. XFast-3 contains a forkhead/winged-helix DNA binding domain and a high affinity SIM, through which it recruits activated Smad2 and Smad4 to the ARE more efficiently than XFast-1. We identify derrière as a good candidate for the endogenous signalling molecule that activates the XFast-3/Smad complex in vivo. Using highly specific morpholino antisense oligonucleotides (Heasman et al., 2000; Nasevicius and Ekker, 2000) to efficiently and specifically inhibit the synthesis of XFast-1 or XFast-3, and hence the formation of ARF1 and ARF2, we identify a major role for ARF1 and ARF2 in controlling the convergent extension movements of gastrulation.

MATERIALS AND METHODS

Cloning of XFast-3 and other DNA constructs

XFast-3 was isolated from a *Xenopus* stage 11 cDNA library in λZAPII using a probe comprising the coding region of *XFast-1* (Chen et al., 1996) as previously described (Howell et al., 1999). The longest positive clone was fully sequenced and the 5′ end of the gene was confirmed by primer extension and RT-PCR. Coding sequences of XFast-3 and XFast-1 were subcloned into pBSK and derivatives of pFTX5 (Howell and Hill, 1997), which encode no tag, a Flag tag or HA tag for preparation of synthetic mRNA, and into EF-HA and EF-Flag for expression in NIH3T3 cells. EF-Flag-XFast-1, mouse activin-βA in pSP64T, *Xenopus* BMP4 in pSP64T, derrière in CS2+, Xnr1 in pSP64T and Xnr2 in pSP64T were as previously described (Howell et al., 1999; Jones et al., 1995; Sun et al., 1999). VegT in pGEMT was generated by isolating the coding sequence by PCR from a *Xenopus* stage 10 cDNA library. The GFP-coding sequence was subcloned into pFTX5.

Embryo manipulations, in situ hybridisation and morpholino oligonucleotide injections

Fertilisation, culture, staging, microinjection of *Xenopus* embryos and animal cap assays were exactly as previously described (Howell and Hill, 1997). Full-length synthetic mRNA for injection was prepared as described (Howell and Hill, 1997) and the amounts of individual mRNAs injected are given in the legend to Figs 5 and 7. In vitro translations were performed as described (Howe et al., 1995).

In situ hybridisation was as described (Germain et al., 2000); probes were as for RNase protections (below).

Morpholino oligonucleotides were obtained from Gene Tools LLC and had the base composition 5′AGTACAGACTGGAGGGGTCTCTCAT3′ (anti-XFast-1); 5′GGTGAAGCCCAAAAGACATGTCAGT3′ (anti-XFast-3) and 5′CCTCTTACCTCAGTTACAATTTATA3′ (human β-globin – control). They were resuspended in sterile water at a concentration of 17 mg/ml (2 mM), centrifuged and injected into single cell embryos at a dose of 10-25 ng per embryo.

Tissue culture, transfections and transcription assays

NIH3T3 cells were cultured and transfected as described (Germain et al., 2000). Transcription assays using the ARE-luciferase reporter were as described (Pierreux et al., 2000).

Bandshift assays and western blotting

For bandshift assays, nuclear extracts from *Xenopus* embryos and transfected NIH3T3 cells were prepared as described (Howell et al., 1999; Pierreux et al., 2000) and whole cell NIH3T3 extracts were as described (Germain et al., 2000). Bandshift assays and supershifts with antibodies against Smad4, Smad2/3 and Flag were as described (Howell et al., 1999; Pierreux et al., 2000). The anti-XFast-1 and anti-XFast-3 antibodies were polyclonal anti-peptide antibodies generated in rabbits against the following peptides (PVATGQSYNHSVQPWPQPWC, XFast-1 and FGLHPWDVAFRPSPPHNLEC, XFast-3). In supershift assays, antibodies were added either alone or with 5 μg of the peptide to which they were raised. All SIM peptides (Germain et al., 2000) used in the competitions had the penetratin sequence (RQIKIWFQNRRMKWKK) at the N terminus, followed by the specific sequences PEVKNAPKDFPPNKTVFDIPVYTGHPGFLA (XFast-3 SIM wild type); PEVKNAPKDFAAAKTVFDIPVYTGHPGFLA (XFast-3 SIM mutant); PLDLNMLRAMPPNKSVFDVLTSHPGDLV (XFast-1 SIM wild type); and PLDLNMLRAMAAAKSVFDVLTSHPGDLV (XFast-1 SIM mutant). Peptides were dissolved in water and used at the amounts indicated in the legend to Fig. 3.

Whole embryo extracts for western blotting were prepared as described (Howe et al., 1995). Western blotting was performed with standard protocols using antibodies against Smad2/3 (as above) or phospho-Smad2 (Faure et al., 2000) or HA (Pierreux et al., 2000).

Isolation of total RNA and RNase protections

Isolation of total RNA from *Xenopus* embryos and RNase protections were as described (Howell et al., 1999). The following antisense probes were as previously described: *Mix.1/Mix.2* and *goosecoid* (Germain et al., 2000), *XFKH1* and *chordin* (Howell and Hill, 1997), *XWnt11* (Tada and Smith, 2000), *shh* (Ekker et al., 1995) and *Xbra*, *EF-1α* and *FGFR* (Howell et al., 1999). Other antisense probes protected nucleotides encoding the following: *XFast-1*, amino acids 473-534; *XFast-3*, 274-323; *XDelta1*, 465-721; *cerberus*, 1-95; *Xlim-1*, 214-333; and *paraxial protocadherin* (*PAPC*) (Kim et al., 1998) amino acids 966-979 and 578 nucleotides of the 3′ UTR.

RESULTS

XFast-3 is a novel *Xenopus* Fast family member

We have previously identified a novel XSmad2/XSmad4β-containing complex, ARF2, that binds the *Mix.2* ARE (Fig. 1A) (Howell et al., 1999). Unlike the XFast-1-containing ARF1, ARF2 is induced by endogenous signalling pathways and is readily detected in uninjected embryos (Fig. 1A). It contains a distinct transcription factor with the same DNA-binding specificity as XFast-1, which we reasoned was another Fast family member.

We screened a stage 11 cDNA library and identified a novel *Xenopus* Fast family member, XFast-3 (Fig. 1B). It is smaller than XFast-1 and shares only three regions of homology with the other known Fast family members (Fig. 1B) (Attisano et al., 2001). Towards the N terminus, the forkhead/winged helix DNA-binding domain is 65% identical between the five family members. Towards the C terminus are two short regions of homology shared by all five family members that are within the Smad interaction domain (SID) identified in XFast-1 (Chen et al., 1997). The more N-terminal of these, which we have named the Fast Motif (FM), has no known function. The most C-terminal region of homology is the SIM (Germain et al., 2000).

XFast-3 forms a transcriptionally active complex at the ARE with endogenous Smad2 and Smad4 in a ligand-dependent manner

Upon TGFβ induction, transfected XFast-1 forms a XFast-1/Smad2/Smad4 complex (ARF1) with endogenous Smads in NIH3T3 cells (Fig. 2A, lanes 1,2) (Germain et al., 2000)). Similarly, a TGFβ-induced XFast-3/Smad2/Smad4 complex (ARF2) is detected in extracts from cells transfected with Flag-

tagged XFast-3 (lanes 6,7). Supershifts with specific antibodies demonstrated that these ARF complexes contained the relevant Flag-tagged Fast transcription factor, Smad2 and Smad4 (lanes 3-5 and 8-10).

Recruitment of active Smads to DNA via XFast-1 generates a transcriptionally active complex (Germain et al., 2000), and the same is true for XFast-3. Both XFast-1 and XFast-3 confer strong TGFβ-dependent transcriptional activation onto an otherwise inactive ARE-luciferase reporter, through their ability to recruit activated Smad2/Smad4 complexes to the ARE (Fig. 2B).

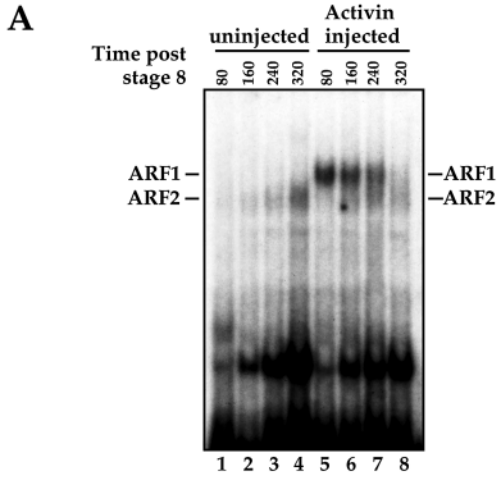
XFast-3 is the endogenous transcription factor component of ARF2

To prove that XFast-3 is the transcription factor component of the endogenous *Xenopus* ARF2 complex, we raised specific polyclonal anti-peptide antibodies against XFast-1 and XFast-3. Endogenous *Xenopus* ARF1, which is detected in extracts from embryos that overexpress activin 80 minutes after stage 8 (Fig. 2C, lane 7) is supershifted by the anti-XFast-1 antibody and not by the anti-XFast-3 antibody (lanes 9 and 11). By contrast, endogenous ARF2, which is readily detected in extracts from uninjected embryos 240 minutes post stage 8 (lane 13), is supershifted only by the anti-XFast-3 antibody (lanes 15 and 17). In both cases, the supershifts were reversed by the peptide to which the antibody had been raised (lanes 10 and 18). Thus, the endogenous ARF2, which is activated in response to endogenous signalling pathways, contains XFast-3, and ARF1, which is detectable only when activin is overexpressed, contains XFast-1 as previously demonstrated (Watanabe and Whitman, 1999).

XFast-3 very efficiently competes with XFast-1 for binding to activated Smads and to the ARE

Even when activin is overexpressed in embryos, the XFast-1-containing ARF1 complex disappears at ~240 minutes post stage 8, coincident with the appearance of the XFast-3-containing complex, ARF2 (Howell et al., 1999), suggesting that either the XFast-1 protein is degraded at this time, or XFast-3 efficiently competes with XFast-1 for activated Smads and the ARE. We tested the latter idea by transfecting NIH3T3 cells with a constant amount of XFast-3 and a titration of XFast-1, or a constant amount of XFast-1 and a titration of XFast-3, checking the actual amount of each XFast expressed by western blotting.

When expressed at equimolar levels (Fig. 3A, lanes 4 or 12, lower panel) XFast-3 forms a complex with activated Smads



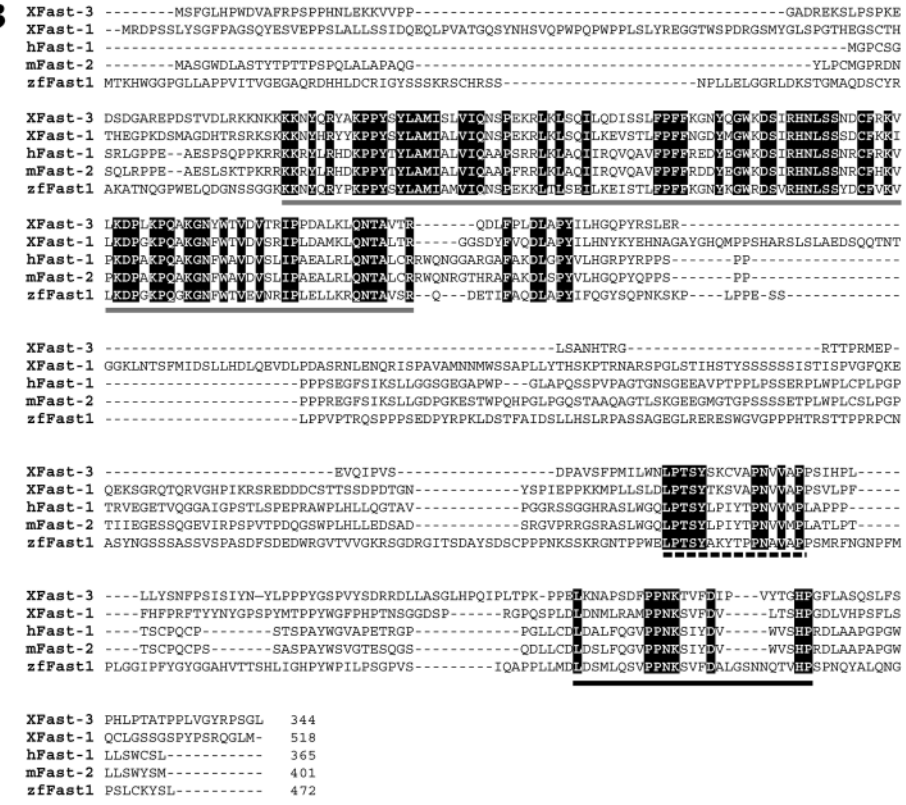


Fig. 1. XFast-3 is a novel member of the Fast family. (A) ARF2 is an ARE-binding complex distinct from the XFast-1-containing ARF1, which appears about 240 minutes post stage 8. Nuclear extracts prepared from uninjected embryos or embryos injected with 200 pg mRNA encoding activin-βA at the times indicated were analysed by bandshift using the ARE as probe. ARF1 is only detected in activin-injected embryos; the faster mobility complex ARF2 is detected in uninjected embryos. (B) The sequence of XFast-3 aligned with the sequences of XFast-1, mouse Fast-2/FoxH1, human Fast-1/FoxH1 and zebrafish FoxH1 (Attisano et al., 2001). Grey underlining, forkhead/winged-helix DNA-binding domain (Kaufmann and Knöchel, 1996); broken line, the Fast motif (FM); unbroken black line, the Smad interaction motif (SIM) (Germain et al., 2000).

and DNA much more readily than does XFast-1. Even when XFast-3 was expressed at barely detectable levels in the western blot, a strong ARF2 complex was detected (lane 3). Indeed ARF2 competes efficiently with ARF1 (e.g. compare lanes 2 and 5) while ARF1 competes very inefficiently with ARF2 (lanes 9-12). This was not explained by Fast-DNA binding affinity as XFast-1 bound DNA in the absence of Smads considerably more strongly than did XFast-3 (data not shown; note the readily detectable levels of XFast-1-DNA complexes in lanes 1-6 and absence of XFast-3/DNA complexes in lanes 7-12). We therefore investigated the relative strength of the Fast SIMs by a peptide competition assay in which SIM peptides disrupt transcription factor-Smad complexes by binding to the activated Smad2 component (Germain et al., 2000). The XFast-3 SIM peptide disrupted the ARF1 complex at a much lower concentration than did the XFast-1 SIM peptide, indicating that the XFast-3 SIM has a much higher affinity for Smad2 than the XFast-1 SIM (Fig. 3B, lanes 3-6 and 11-14). Moreover, the XFast-3 SIM peptide, but not the XFast-1 SIM peptide disrupted the ARF2 complex (Fig. 3C, lanes 3-5 and 9-11). Controls with mutant peptides indicated that the peptides were specific (Fig. 3B,C). The strong interaction between XFast-3 and the Smads in ARF2 is also evident from the observation that ~10-fold more XFast-3 SIM peptide was needed to disrupt ARF2 than ARF1 (compare Fig. 3B lane 6 with 3C lane 4).

Thus, when XFast-3 and XFast-1 are co-expressed, XFast-3 preferentially binds activated Smads and forms the ARF2 complex at the ARE.

XFast-3 has a narrow window of expression during early- and mid-gastrulation

In *Xenopus* embryos, XFast-3 mRNA is expressed only during gastrulation, coincident with the appearance of ARF2 (Fig. 4A; stage 10.25-11). This pattern contrasts with that of XFast-1, which is present as a maternal message, and is maintained zygotically until it finally disappears between stage 18 and 21 (Fig. 4A) (Chen et al., 1996).

The spatial expression pattern of XFast-1 and XFast-3 was assessed in stage 10.25 embryos bisected through the dorsal lip. Both Fast family members have similar expression patterns at this stage being most highly expressed in the animal cap (prospective ectoderm) and prospective mesoderm (Fig. 4B). This expression pattern is almost mutually exclusive with that of another Smad2-interacting transcription factor, Mixer, which is expressed in deep mesoendodermal cells (Hill, 2001).

Complexes containing XFast-3 are preferentially activated by derrière and VegT

ARF2 forms in response to endogenous ligands (Fig. 1A)

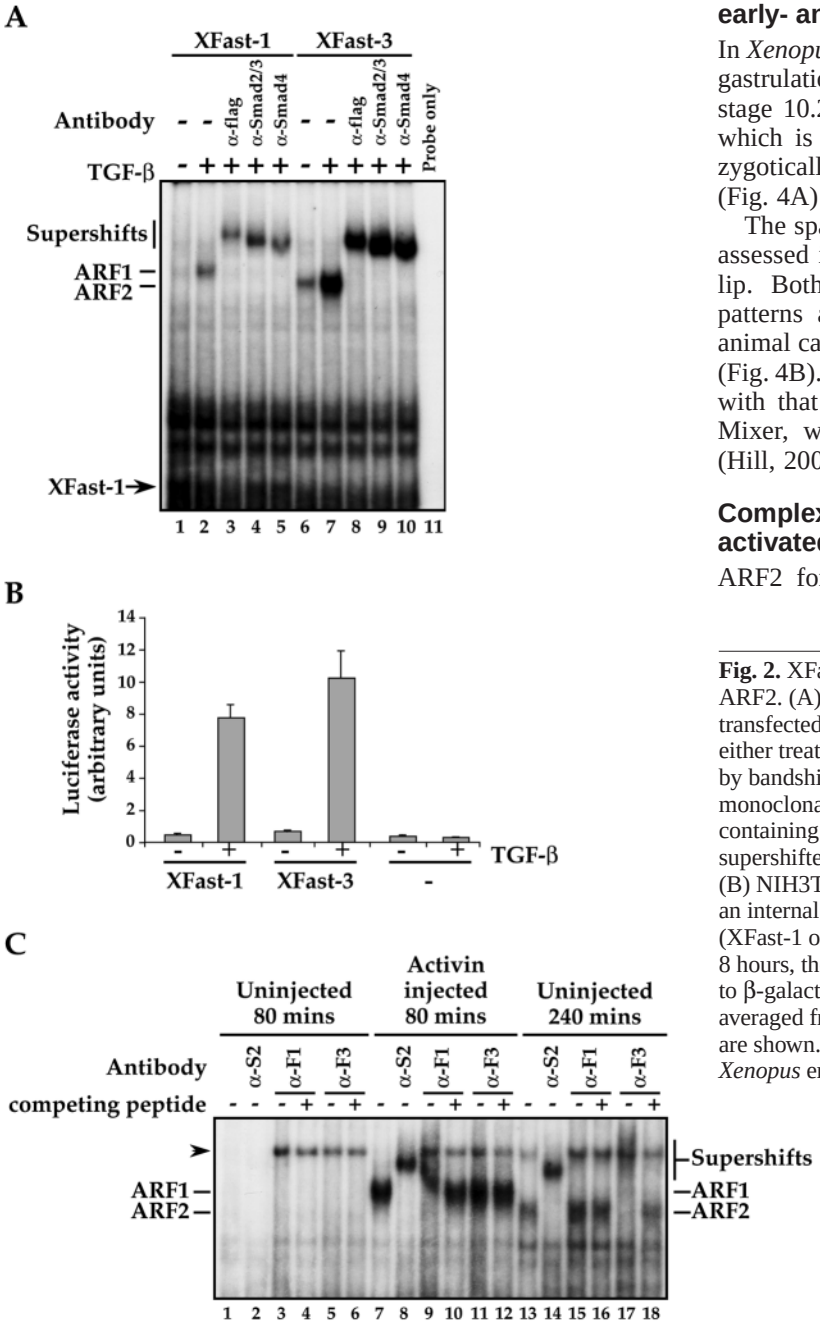


Fig. 2. XFast-3 is the transcription factor component of endogenous ARF2. (A) Whole cell extracts were prepared from NIH3T3 cells transfected with either Flag-tagged XFast-1 or XFast-3 that were either treated or not with TGFβ1 as indicated. Extracts were analysed by bandshift assay on the ARE and complexes supershifted with monoclonal antibodies against Flag, Smad2/3 or Smad4. The Smad-containing complexes ARF1 and ARF2 are indicated, as are the supershifted complexes and the complex of XFast-1 with DNA. (B) NIH3T3 cells were transfected with ARE-luciferase, EF-*lacZ* as an internal control and plasmids expressing transcription factor (XFast-1 or XFast-3), as indicated. Cells were treated with TGFβ1 for 8 hours, then harvested and luciferase activity was measured relative to β-galactosidase activity from the internal control. The data are averaged from six independent experiments and standard deviations are shown. (C) Nuclear extracts were prepared from uninjected *Xenopus* embryos or embryos overexpressing activin at the times indicated post stage 8 and analysed by bandshift assay on the ARE. Antibodies against Smad2 (S2), XFast-1 (F1) or XFast-3 (F3) were included in the bandshift reaction where indicated, alone or with 5 μg peptide to which the antibody had been raised. ARF1 and ARF2 are indicated, as are supershifted complexes. Arrowhead indicates complex resulting from the XFast-1/3 antisera alone binding the probe; competing peptides have no effect on this complex.

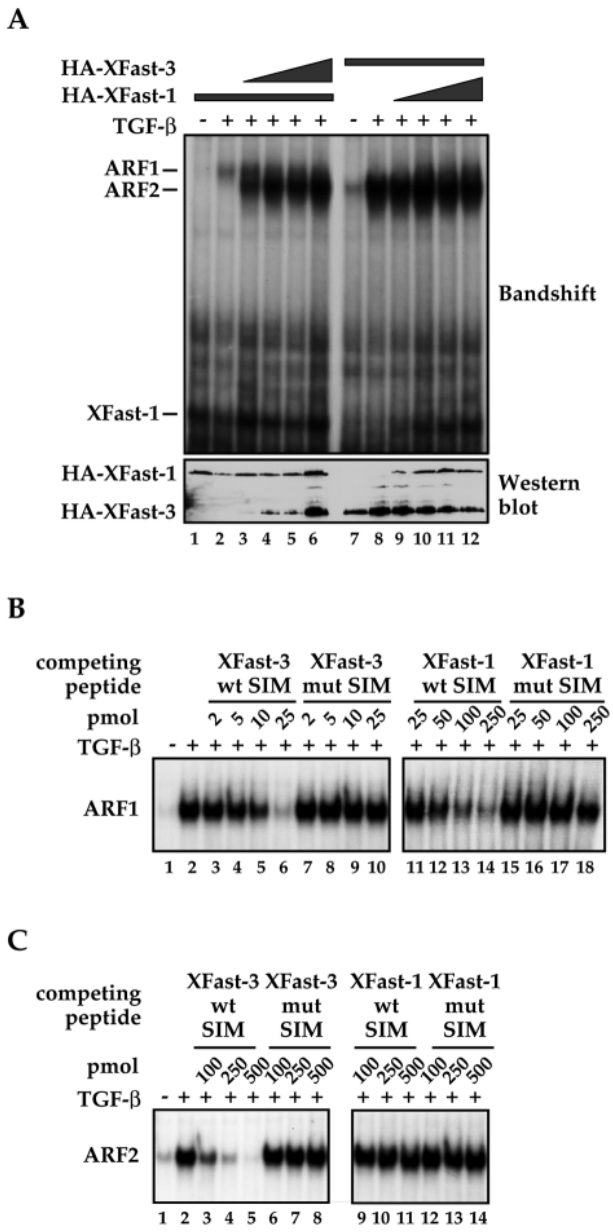


Fig. 3. Efficient ARF2 competition with ARF1 is explained by the high affinity SIM in XFast-3. (A) XFast-3 competes very efficiently with XFast-1 for binding activated Smads and the ARE. NIH3T3 cells were transfected with 1 μg HA-tagged XFast-1 alone or together with 0.25, 0.5, 0.75 or 1 μg HA-tagged XFast-3 or with 1 μg of XFast-3 and the same titration series of HA-tagged XFast-1. Cells were induced (or not) with TGFβ1 as indicated. Whole-cell extracts were prepared and analysed for ARF1 and ARF2 by bandshift on the ARE (upper panel) or western blotted with anti-HA antibody (lower panel). ARF1 and ARF2 are indicated, as is the complex of XFast-1 with DNA. No XFast-3-DNA complex is detected. (B,C) A peptide corresponding to the XFast-3 SIM disrupts ARF1 and ARF2 much more efficiently than a peptide corresponding to the XFast-1 SIM. NIH3T3 cells were transfected with Flag-tagged XFast-1 (B) or XFast-3 (C) and incubated with TGFβ1 to induce formation of ARF1 or ARF2, respectively, in a bandshift assay on the ARE. Increasing amounts (pmoles) of wild-type (wt) or mutant (mut) SIM peptides corresponding to the SIMs of XFast-3 or XFast-1 were included in the bandshift reactions as indicated.

(Howell et al., 1999). To identify possible candidates, we assayed whether Xnr1 or Xnr2 (Jones et al., 1995), derrière (Sun et al., 1999), activin itself or VegT, the T-box transcription factor that regulates a number of these activin-like ligands (Zhang et al., 1998), could activate the formation of ARF2. BMP4, which does not activate Smad2, was used as a negative control. ARF1 is activated only at the early time point by overexpression of activin and Xnr1 and weakly by Xnr2 (Fig. 5A, top panel), while ARF2 is activated by endogenous signals at the later time point (bottom panel, lane 1) and by overexpression of derrière and VegT (lanes 5 and 11). All the inducers were active as demonstrated by their ability to induce mesoendodermal marker genes (data not shown).

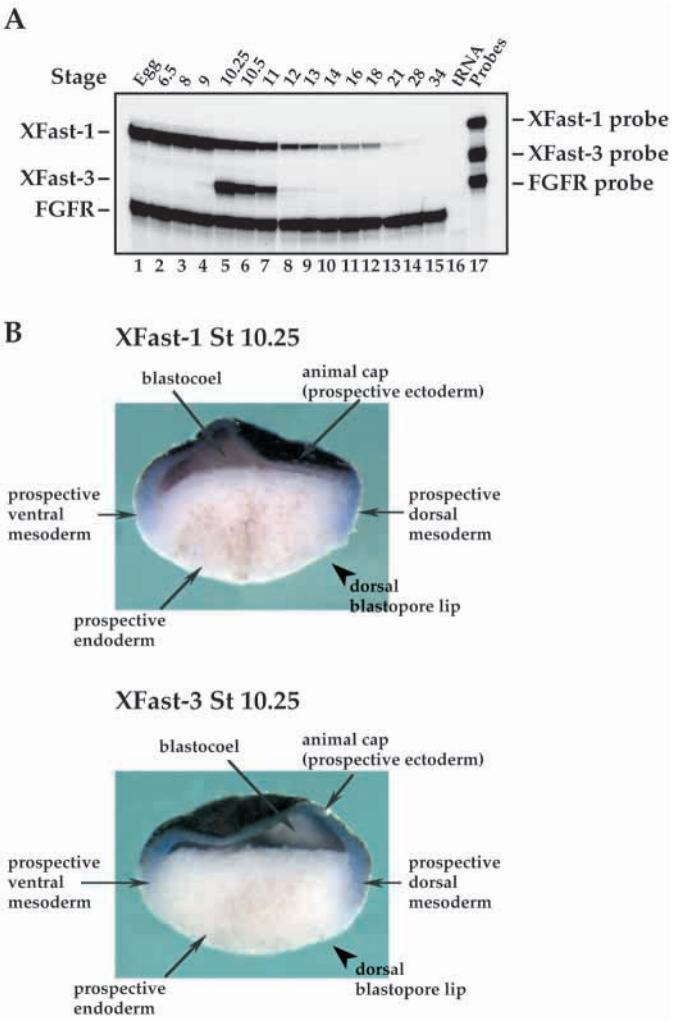


Fig. 4. XFast-3 is expressed in a narrow window at early and mid-gastrulation. (A) Total RNA was extracted from embryos at the times indicated and analysed by RNase protection using probes against XFast-1, XFast-3 or FGFR (loading control). The protected fragments are indicated, as are the undigested probes. The lane marked tRNA is a control for probe digestion. (B) XFast-1 and XFast-3 are expressed strongly in the animal cap and in the prospective mesoderm of stage 10.25 embryos. The embryos were bisected through the dorsal lip before in situ hybridisation using probes against XFast-1 or XFast-3. Specific staining is blue, and is strongest in the prospective mesoderm and ectoderm. The dark brown colour is the pigmented animal cap.

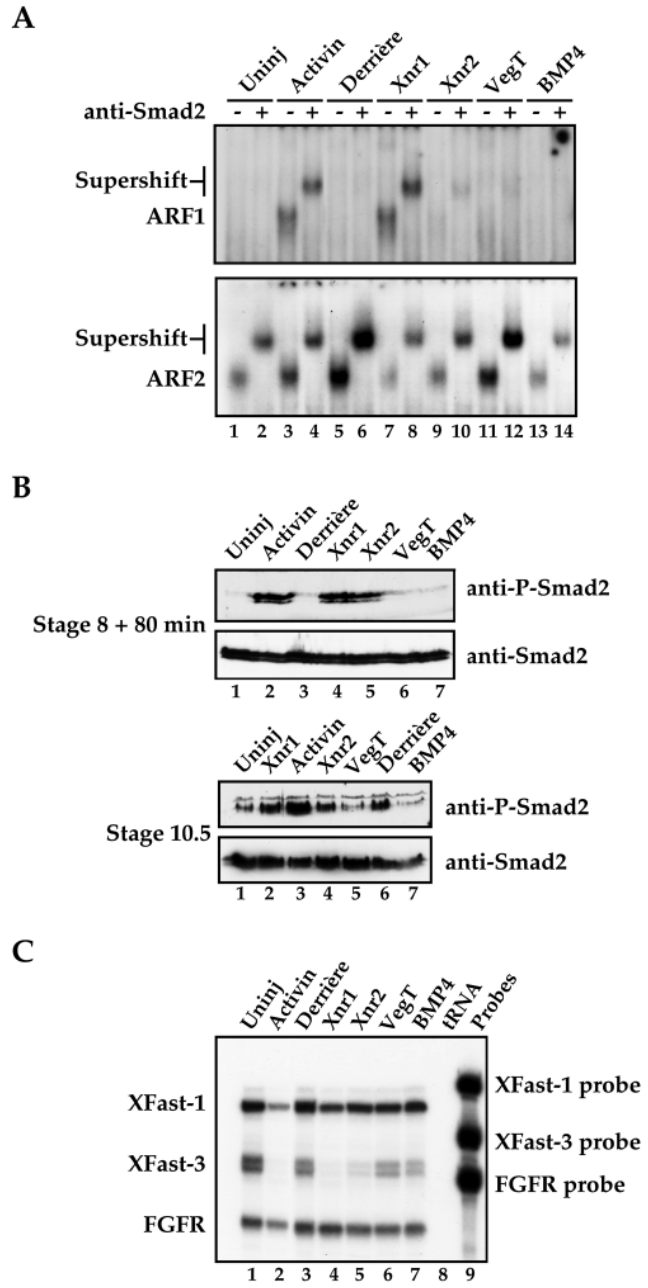
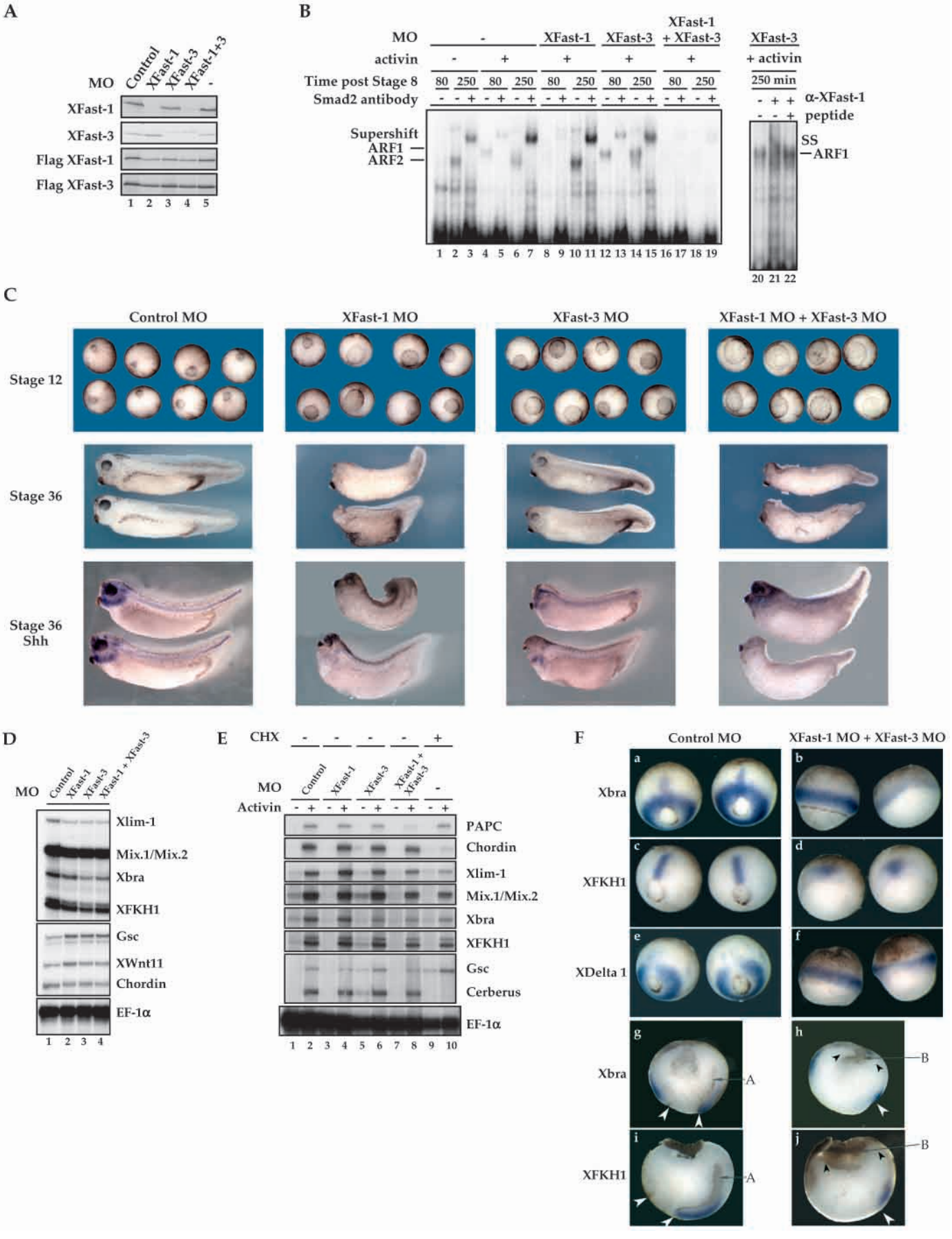


Fig. 5. ARF1 and ARF2 are activated by distinct signalling molecules. (A) Single cell embryos were injected with 200 pg synthetic RNA encoding activin βA or 1.5 ng RNA encoding derrière, Xnr1, Xnr2,VegT or BMP4. Embryos were cultured until 80 minutes post stage 8 (to detect ARF1, upper panel) or 240 minutes post stage 8 (to detect ARF2, lower panel). Nuclear extracts were prepared and assayed by bandshift assay on the ARE in the presence or absence of anti-Smad2/3 antibody. (B) Whole embryo extracts were prepared from injected embryos at 80 minutes post stage 8 (upper panels) or stage 10.5 (lower panels) and analysed by western blotting with an antibody against activated phosphorylated Smad2 (anti-P-Smad2), or Smad2/3. *Xenopus* embryos contain no Smad3 at this time (Howell et al., 2001). The upper band is full-length Smad2; the lower band is the spliced isoform missing exon 3 in the MH1 domain (Faure et al., 2000). (C) XFast-3 expression is inhibited by activin, Xnr1 or Xnr2. Total RNA, prepared from injected embryos at stage 10.5, was analysed by RNase protection using probes against *XFast-1, XFast-3* or *FGFR*. Protected fragments are as indicated.

As ARF complex formation requires Smad2 phosphorylation, we investigated the ability of the inducers to induce Smad2 phosphorylation at the two time points by western blotting (Faure et al., 2000). At 80 minutes post stage 8, Xnr1 and activin, and to a lesser extent Xnr2, activated Smad2, whereas VegT, derrière and BMP4 did not (Fig. 5B, top panels). By stage 10.5, levels of phosphorylated Smad2 in uninjected embryos were elevated as a result of endogenous activin-like signalling pathways (bottom panels, lane 1). Overexpression of Xnr1, Xnr2, derrière and activin all efficiently induced the level of phosphorylated Smad2 at this stage (bottom panels).

Thus, although derrière mRNA was injected at the one-cell stage, it only activates Smad2 at stage 10.5 (Fig. 5B) (Lee et

Fig. 6. ARF1 and ARF2 are required for convergent extension movements of gastrulation. (A,B) Morpholinos (MOs) against either XFast-1 or XFast-3 specifically block translation in vitro and in vivo. (A) Synthetic mRNAs corresponding to native XFast-1 and XFast-3 or Flag-tagged XFast-1 and XFast-3 (as indicated) were translated in reticulocyte lysate in the presence of 0.2 mM of the indicated morpholino and [^{35}S]-methionine. Translation products were separated by SDS-PAGE and visualised by autoradiography. (B) Nuclear extracts were prepared at the times shown from either uninjected embryos or embryos injected with 200 pg activin βA mRNA and/or 10 ng of MOs against XFast-1, XFast-3, or both. Equal amounts of extract were analysed for the presence of ARF1 or ARF2 by bandshift using an ARE probe. Anti-Smad2/3 or anti-XFast-1 antibodies were included in the reaction as indicated to confirm the complexes as ARF1 and ARF2. The ARF1, ARF2 and supershifted (SS) complexes are indicated. The reduction of ARF1 and ARF2 in the embryos injected with MOs against XFast-1 and XFast-3 is 10- to 20-fold. (C) Depletion of ARF1, ARF2 or both has a dramatic effect on embryo development. Embryos were injected with 50 ng of control MO directed against human β-globin, or 25 ng MO against XFast-1 or XFast-3 with 25 ng control, or 25 ng each of XFast-1 and XFast-3 MOs. Embryos were sampled when those injected with control MO reached stage 12.5 (upper panel) and stage 36 (middle panel). A medium phenotype (see Table 1) is shown in each case. Lower panel shows an in situ hybridisation on stage 36 embryos with a *shh* probe. Specific staining is blue. (D,E) Depletion of ARF1 or ARF2 or both partially inhibits expression of a subset of mesoendodermal genes. (D) Embryos injected with MOs as in B were cultured until those injected with control MO reached stage 10. (E) Animal caps were dissected from MO-injected embryos at stage 8 and induced with activin (40 ng/ml) until control embryos reached stage 10.25. Total RNA was prepared and analysed by RNase protection using the probes indicated. In E, the last two lanes correspond to animal caps dissected from uninjected embryos that had been treated ± activin in the presence of 5 μg/ml cycloheximide (CHX) (Howell and Hill, 1997) to indicate which genes were direct targets of the activin signalling pathway. (F) Depletion of ARF1 and ARF2 inhibits convergent extension movements of gastrulation. (a-f) Embryos injected with 50 ng control MO or 25 ng each of MOs against XFast-1 and XFast-3 were fixed when those injected with control MO reached stage 12 and whole-mount in situ hybridisation was used to detect the transcripts of *Xbra*, *XFKH1* and *XDelta1* as indicated. Specific staining is blue. Dorsal side is uppermost in all cases. (g-j) Midsagittal halves of embryos injected with control or XFast1 and XFast-3 MOs and stained for *Xbra* or *XFKH1* transcripts. In g and i, the archenteron (A) is indicated, and the extent of the yolk plug by the white arrowheads. In h and j, the white arrow indicates dorsal blastopore lip. No archenteron has formed in these embryos. The blastocoel (B) has been squeezed as a result of some migration of mesoendoderm (black arrowheads).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ARF2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
XFast-3
+ activin
250 min
α-XFast-1
peptide
SS
ARF1
20 21 22
C
Control MO
XFast-1 MO
XFast-3 MO
XFast-1 MO + XFast-3 MO
Stage 12
Stage 36
Stage 36
Shh
D
MO
Control
XFast-1
XFast-3
XFast-1 + XFast-3
Xlim-1
Mix.1/Mix.2
Xbra
XFKH1
Gsc
XWnt11
Chordin
EF-1α
1 2 3 4
E
CHX
MO
Control
XFast-1
XFast-3
XFast-1 + XFast-3
-
Activin
PAPC
Chordin
Xlim-1
Mix.1/Mix.2
Xbra
XFKH1
Gsc
Cerberus
EF-1α
1 2 3 4 5 6 7 8 9 10
F
Control MO
XFast-1 MO + XFast-3 MO
Xbra
XFKH1
XDelta 1
Xbra
XFKH1
a
b
c
d
e
f
g
h
i
j
A
B
A
B

al., 2001). Consequently derrière (and also VegT, which activates derrière) (Sun et al., 1999), only activates ARF2, which contains the zygotic transcription factor XFast-3. By contrast, Xnr1, Xnr2 and activin all induce phosphorylation of Smad2 by 80 minutes post stage 8 and thus preferentially activate ARF1, which contains maternal XFast-1. Xnr1, Xnr2 and activin also activate Smad2 at the time at which ARF2 appears, but do not induce its formation. This is because they downregulate *XFast-3* mRNA, and presumably also XFast-3 protein (Fig. 5C).

Thus, ARF1 and ARF2 are preferentially activated by distinct signalling molecules, and the data indicate that derrière is a good candidate for the endogenous activator of ARF2.

Morpholino oligonucleotides directed against XFast-1 or XFast-3 specifically 'knockdown' levels of these molecules in the developing embryo

To determine the role of these two ARF complexes in early *Xenopus* embryos, we used specific morpholino (MO) antisense oligonucleotides (Heasman et al., 2000; Nasevicius and Ekker, 2000; Summerton and Weller, 1997; Winklbauer et al., 2001) to 'knockdown' levels of their constituent transcription factors, XFast-1 or XFast-3. In all experiments, a human β-globin MO was used to rule out any nonspecific effects of morpholinos per se (control-MO) and we ensured that the total MO concentration was constant for each manipulation.

Morpholinos designed against either XFast-1 or XFast-3 mRNA sequences specifically inhibited the in vitro translation of their respective cognate mRNAs but not vice versa (Fig. 6A, upper two panels, lanes 2, 3 and 4). N-terminally epitope-tagged versions of either XFast-1 and XFast-3 mRNAs, which cannot bind to their respective morpholinos, are resistant to translational inhibition (Fig. 6A, lower two panels).

We then tested the specificity and efficacy of the morpholinos in vivo using a bandshift assay in which the endogenous ARF1 complex (Fig. 6B, lane 4) is easily distinguished from the endogenous ARF2 complex (lanes 2 and 6) by its time of appearance and relative mobility. As we have demonstrated, bandshift assays are highly sensitive (see Fig. 3A lane 3; compare western blot and bandshift) and, when probe is in large excess, quantitative. Injection of one-cell embryos with the XFast-1 MO severely reduced the level of ARF1 in activin-injected embryos at 80 minutes (Fig. 6B compare lanes 4 and 8). This was absolutely specific for XFast-1, as levels of the ARF2 complex were not reduced; in fact they were stronger (compare lanes 6 and 10). Similarly, when embryos were injected with the XFast-3 MO, ARF1 was undiminished (compare lane 4 with 12), ARF2 was depleted and, moreover, was replaced by ARF1 (compare lanes 6 and 14), as confirmed by its ability to be supershifted with the anti-XFast-1 antibody (lanes 20-22). The reappearance of ARF1 at the late time point when XFast-3 synthesis is inhibited by MO injection provides evidence for our view that the replacement of ARF1 with ARF2 normally observed at 250 minutes post stage 8 (lanes 4 and 6) is due to XFast-3 competing efficiently with XFast-1 for the active Smads and the ARE. It follows from these results that ARF1 might compensate for loss of ARF2, but not vice versa (see below).

When XFast-1 and XFast-3 MOs were injected together, the formation of both ARF1 and ARF2 was dramatically reduced (10-20 fold; lanes 16-19).

Table 1. Phenotypes arising from anti-XFast-1 (F1) and XFast-3 (F3) MO injection

MO injection	Wild type (%)	Weak (%)	Medium (%)	Strong (%)	Other (%)
Stage 12*					
MO control (*n*=128)	87.5	0	12.5	0	0
MOF1 (*n*=119)	47.5	0	25	19.5	0
MOF3 (*n*=133)	67.7	0	23.3	9	0
MOF1+F3 (*n*=150)	18.7	0	23.3	58	0
Stage 27†					
MO control (*n*=120)	75	15	5	5	0
MOF1 (*n*=119)	3.7	33.9	44	26.6	0.9
MOF3 (*n*=128)	14§	25.8	53.9	4.7	1.6
MOF1+F3 (*n*=137)	0	43	21.9	33.6	1.5
Stage 35‡					
MO control (*n*=120)	67.5	9.2	8.3	15	0
MOF1 (*n*=109)	3.7	0	27.5	68.8	0
MOF3 (*n*=126)	9.5§	19.8	59.5	7.9	3.2
MOF1+F3 (*n*=126)	5.1§	12.4	25.6	56.9	0

*Strong, equatorial blastoporal pigment line only; medium, blastopore wider, diameter similar to stage 11.

†Strong, very short and malformed axis, almost no head structures, epidermal disintegration; medium, short axis, some head structure but truncated, some embryo disintegration; weak, reduced head structure, axis slightly shorter than wild type but usually kinked, dorsal fin reduced.

‡Strong, almost complete disintegration or mostly disintegrated with very short axis and no head; medium, short axis usually kinked, some disintegration, truncated head; weak, reduced dorsal fin and melanophores, nearly normal length but kinked axis.

§Phenotypically wild type but unresponsive to stimuli.

Thus, the morpholino oligonucleotides specifically and efficiently knockdown synthesis of XFast-1 and XFast-3 in vitro and in vivo and consequently reduce levels of endogenous ARF complexes 10- to 20-fold without nonspecific toxicity.

Phenotypic effects of ARF depletion

Depletion of either or both ARF complexes produced embryos exhibiting a range of overlapping phenotypes. At all developmental stages, ARF2 depletion alone produced the weakest phenotypes, as might be predicted if ARF1 can compensate for a loss of ARF2 (see Fig. 6B), whereas the effect of ARF1 depletion was more severe. Depletion of both gave an additive effect and the most severe phenotypes. A typical experiment is summarised in Table 1. Embryos injected with the control MO are phenotypically wild type.

Initiation of blastoporal pigment line formation on the dorsal side was not inhibited, although in the case of embryos depleted of ARF1 or ARF2 and more obviously in those depleted of both, these lips were displaced towards the animal pole (Fig. 6C,F). In embryos depleted of ARF1 or ARF2, subsequent blastopore closure was slower than in control-MO injected embryos and very delayed, and, in some cases, virtually abolished in embryos depleted of both (Fig. 6C, top panel and data not shown). These latter embryos were also unable subsequently to fuse neural folds and often disintegrated (not shown). By stage 36, embryos depleted of ARF2 tended to have shorter axes (less than 80% of wild type embryos) that were kinked with reduced dorsal fin and some reduced head structures (except cement gland), and reduced numbers of melanophores (Fig. 6C, middle panel, Table 1). Those depleted of ARF1 showed more extreme body axis shortening and loss of head structures as well as reduced dorsal

fin and melanophores (Fig. 6C, middle panel). Embryos depleted of both ARF1 and ARF2 exhibited the most severe phenotypes. In the most severely affected, virtually no axial structures formed and the embryos disintegrated through loss of epithelial integrity (Table 1). The rest of the ARF1/ARF2-depleted embryos exhibited phenotypes similar to those observed in embryos depleted of ARF1 or ARF2 (Table 1; Fig. 6C).

The loss of axial structures upon depletion of ARF1, ARF2 or both is demonstrated by the pattern of *sonic hedgehog* (*shh*) staining. In control MO-injected embryos, *shh* is expressed along the notochord and floor plate. Levels of *shh* are very much reduced in embryos depleted of ARF1 and/or ARF2. Somites were formed in all cases as demonstrated by actin expression; however none of the embryos depleted of ARF1 and/or ARF2 could respond to mechanical stimuli (Table 1; data not shown).

Depletion of ARF1 and ARF2 has a severe effect on gastrulation movements

As the length of the embryonic axis is determined in part by the convergent extension movements of gastrulation, we analysed the effect of ARF depletion at these early developmental stages in more detail, initially investigating the expression of a panel of early mesoendodermal genes (Fig. 6D). In no case did we observe complete loss of induction of gene expression. When the embryos injected with control MO had reached stage 10, embryos injected with either XFast-1 or XFast-3 MOs or both showed reduced expression of *Xlim-1*, *Xbra* and *XFKH1*, while levels of *Mix.1/Mix.2*, *XWnt11*, *chordin* or the ubiquitously expressed *EF-1α* were unaffected and *goosecoid* expression was actually increased (Fig. 6D). Similarly, in activin-induced animal caps only relatively small effects of depletion of ARF1 and/or ARF2 were seen (Fig. 6E).

A more dramatic effect of ARF1/ARF2 depletion was on the spatial pattern of expression of these mesoendodermal genes, which indicated that gastrulation movements were impaired (Fig. 6F). Control MO-injected embryos gastrulate normally, closing the blastopore and extending along the dorsal midline as a result of the convergent extension movements. *Xbra* and *XFKH1* are expressed on the extended dorsal midline (and in the case of *Xbra*, in the circumblastoporal mesoderm), *XDelta1* is excluded from the dorsal midline, but expressed in patches in the neural plate (Fig. 6F, panels a,c,e,g,i) (Dirksen and Jamrich, 1992; Henrique et al., 1995; Ruiz i Altaba and Jessell, 1992; Smith et al., 1991). By contrast, embryos of the same age depleted of both ARF1 and ARF2 expressed *Xbra* and *XDelta1* throughout the marginal zone although it is displaced towards the animal pole (Fig. 6F, panels b,f,h). In the ARF1/ARF2-depleted embryos, *XFKH1* is dorsally expressed, but in a patch, as opposed to along the extended dorsal axis (Fig. 6F, compare panel c with d and i with j).

Midsagittal sections of control MO-injected embryos indicated that gastrulation has proceeded normally to form the archenteron (Fig. 6F, panels g,i), whereas in the ARF1/ARF2-depleted embryos the midsagittal sections suggested that no convergent extension movements had occurred and no archenteron had formed (Fig. 6F, panels h,j). Note that not all gastrulation movements are affected in the ARF-depleted embryos, as these embryos had undergone significant mesoendoderm migration (Fig. 6F, panels h,j, black arrows).

ARF1 and ARF2 are required for activin-induced convergent extension movements in animal cap explants

We tested directly whether depletion of ARF1 and ARF2 prevents the convergent extension movements of gastrulation. Animal caps cut at stage 8 from embryos injected with the control MO extended dramatically when treated with activin (Fig. 7A). By contrast, those injected with XFast-1 and XFast-3 MOs, and thus depleted of both ARF1 and ARF2, did not extend at all (Fig. 7A).

Extension of animal caps treated with activin at stage 8 is primarily dependent on XFast-1, as no XFast-3 is expressed at stage 8 (Fig. 4A). To prove that the effects of the morpholinos are specifically due to depletion of the XFasts, we confirmed that injection of the XFast-1-MO inhibited animal cap extension, but injection of the XFast-3-MO did not (Fig. 7B, panels a-d). Furthermore, the inhibition of activin-induced extension of XFast-1-depleted animal caps could be efficiently rescued by morpholino-resistant versions of XFast-1 or XFast-3 mRNA (Fig. 7B, panels e and f). This was specific, as control GFP mRNA could not rescue the phenotype (Fig. 7B, panel d). This further confirmed that the cap phenotype was due to the depletion of XFast-1 (and thus ARF1). As previously shown by Whitman and colleagues (Watanabe and Whitman, 1999), overexpression of full length XFast-1 is deleterious to normal embryonic development, and thus it was not possible to rescue the whole embryo phenotypes.

Taken together with the embryo phenotypes, the data indicate that in whole embryos, ARF1 and ARF2 are required for the convergent extension movements of gastrulation.

DISCUSSION

XFast-3 is a new member of the Fast family

We have identified XFast-3 as an important new member of the Fast family of transcription factors. Like XFast-1, XFast-3 forms a complex with activated XSmad2 and XSmad4β at the ARE. It recruits activated Smads through its SIM and its transcriptional activity depends on complex formation with activated Smads. However its affinity for the activated Smads is much higher than that of XFast-1 and its temporal expression pattern is distinct from that of XFast-1. What role, therefore, does XFast-3 play during gastrulation?

From our morpholino experiments, it is clear that inhibiting the expression of XFast-3 produces an embryonic phenotype on its own, albeit a weaker phenotype than for XFast-1-depleted embryos. XFast-1 and XFast-3 are therefore not completely functionally redundant. It is also evident that XFast-1 compensates for the loss of XFast-3 better than XFast-3 compensates for the loss of XFast-1, probably as a result of their different temporal expression patterns.

The lack of redundancy suggests there may be a subset of XFast-3/ARF-specific target genes whose expression cannot be rescued by XFast-1/ARF1. Indeed, site selections with XFast-1 and XFast-3 have revealed subtle differences in site preference for both the transcription factors in isolation and for the ARF complexes containing them (M. H. and C. S. H., unpublished). Alternatively, as the phenotype of the XFast-3

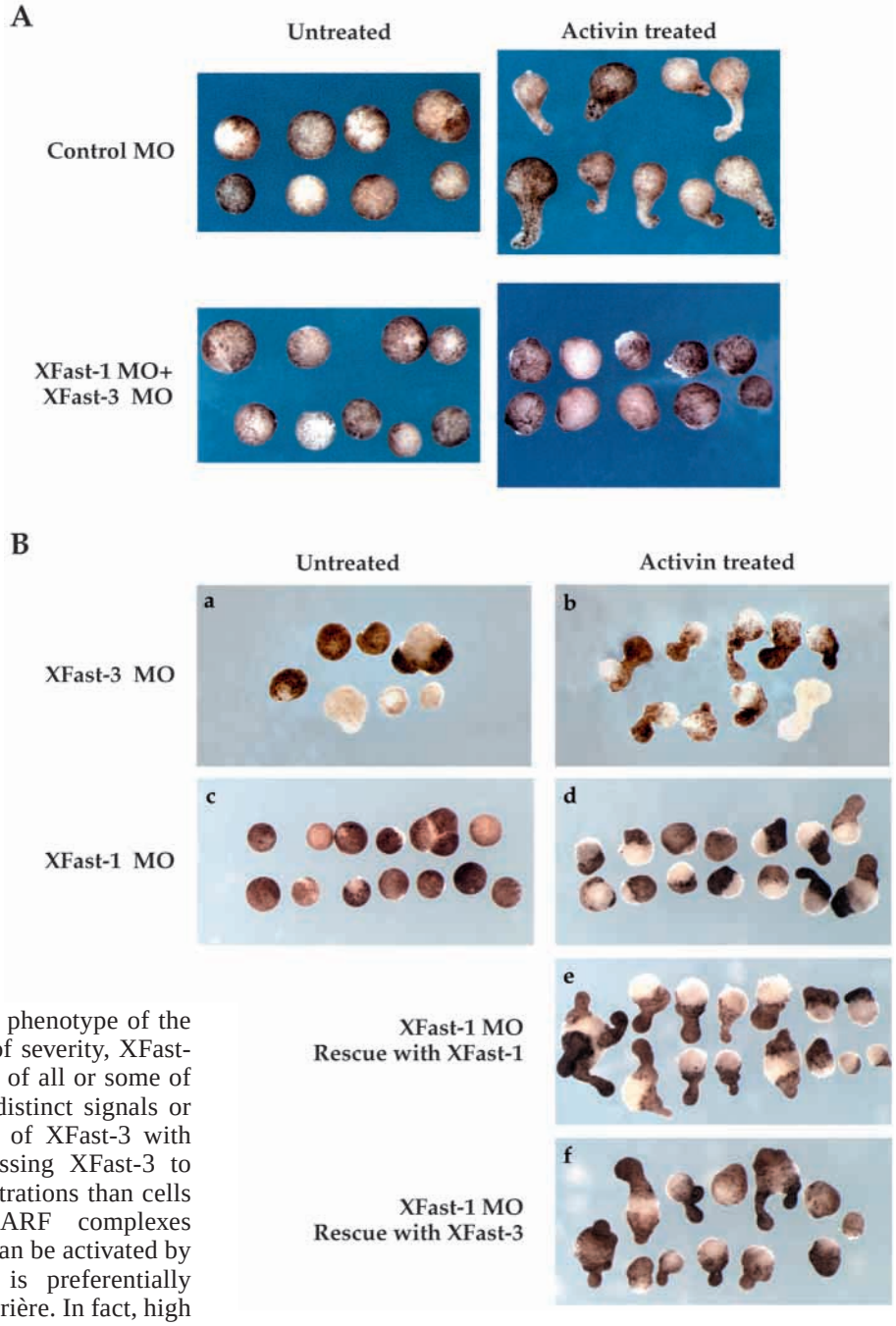

Fig. 7. Depletion of ARF1 and ARF2 specifically inhibits convergent extension movements in activin-induced animal caps. (A) Animal caps cut at stage 8 from embryos injected with 50 ng control MO or 25 ng each of XFast-1 and XFast-3 MOs were either untreated (left panels) or treated with activin (20 ng/ml; right panels) until control embryos reached stage 13. (B) Expression of XFast-1 or XFast-3 rescues the inhibition of activin-induced convergent extension movements in XFast-1-depleted animal caps. Animal caps cut at stage 8 from embryos injected with 25 ng XFast-3 MO and 1 ng control GFP mRNA (a,b) or 25 ng XFast-1 MO with either 1 ng control GFP mRNA (c,d), Flag-tagged XFast-1 mRNA (e) or Flag-tagged XFast-3 mRNA (f) were either untreated (left panels) or treated with activin (40 ng/ml; right panels) until control embryos reached stage 13. Note that in a,c,d-f, some of the caps have stuck together.

knockdown embryos only differs from the phenotype of the XFast-1 knockdown embryos by degrees of severity, XFast-3 may be required for maximal expression of all or some of the XFast-1 target genes, in response to distinct signals or levels of signal. The stronger interaction of XFast-3 with activated Smads will enable cells expressing XFast-3 to respond to a wider range of ligand concentrations than cells expressing only XFast-1. Moreover ARF complexes containing these two transcription factors can be activated by different activin-related ligands: ARF1 is preferentially activated by Xnr1 and activin, ARF2 by derrière. In fact, high levels of Xnr1/2 and activin inhibit XFast-3 expression, ensuring a distinction between those cells responding to Xnr1/2 signalling through XFast-1 and those responding to derrière through XFast-3. These issues will be resolved by analysing the genes whose expression is XFast dependent, and identifying XFast-1- or XFast-3-specific targets.

What is the role of maternal XFast-1? XFast-1 binds DNA in the absence of activated Smads and interacts with activated Smads relatively weakly. By contrast, XFast-3, which appears later, does not efficiently bind DNA alone, but instead forms a very strong complex with activated Smads through its high affinity SIM. We speculate that the binding of maternal XFast-1 to DNA in the absence of activated Smads may be functionally significant in vivo. It may act with co-repressors to keep genes repressed in the absence of ligand, analagous to the Hedgehog, Wnt and Notch signal transduction pathways. Upon ligand induction XFast-1 would be converted to an activator by binding the activated Smads and/or by being replaced by the XFast-3/Smad complex, ARF2.

The phenotype of ARF1 and ARF2 'knockdown' embryos; comparisons with mice and zebrafish

We have demonstrated that stage 36 embryos depleted of ARF1 and/or ARF2 exhibit a range of phenotypes, which include shortened body axes, disrupted dorsal axial structures, anterior truncations (although the cement gland is still present), reduced dorsal fin and melanophores. The reduced axis length is

explicable by our demonstration of reduced convergent extension movements during gastrulation in the ARF-depleted embryos, and we are currently investigating whether the reduced dorsal fin and melanophores are a secondary defect perhaps as a result of inhibition of some aspect of neural crest cell development.

The phenotype of mice deficient in the single known mouse Fast (FoxH1) has been reported (Hoodless et al., 2001; Yamamoto et al., 2001) and shows a number of similarities to those described above for *Xenopus* ARF-depleted embryos. The *FoxH1*$^{-/-}$ embryos can be classified into three phenotypes: type I embryos have axial defects and lack a definitive node and notochord; type II embryos lack anterior structures (such as the head fold), but possess posterior structures with midline defects; and type III embryos lack structures derived from the embryo proper as a result of defects in anteroposterior patterning. Details of these phenotypes suggest a role for Fast/FoxH1 in gastrulation movements in mice and led Yamamoto and colleagues to conclude that loss of Fast-1/FoxH1 in mouse embryos affected Nodal-dependent anteroposterior patterning, and elongation and patterning of the primitive streak, though not Nodal-dependent mesoderm induction (Yamamoto et al., 2001).

A zebrafish Fast family member has also been cloned and mutant embryos [*schmalspur* (*sur*)] that lack functional maternal and zygotic Fast-1 have been characterised (Pogoda et al., 2000; Sirotkin et al., 2000). The MZ*sur* embryos display defects in dorsal axial structures, shortened body axis, anterior truncations of the brain, but form endoderm and paraxial mesoderm normally (Pogoda et al., 2000; Sirotkin et al., 2000), a phenotype similar to the type I *FoxH1*$^{-/-}$ mouse embryos (Yamamoto et al., 2001), and reminiscent of the ARF-depleted *Xenopus* embryos. However, to what extent the *sur* mutant phenotype results from impairment of gastrulation movements remains to be determined.

Previous work has investigated the function of XFast-1 in *Xenopus* embryos by overexpression of a dominant-negative engrailed repressor-XFast-1 fusion (FAST-En^{R}) (Watanabe and Whitman, 1999). These studies identified many of the phenotypic effects we have described using our morpholino approach such as delayed blastopore closure and later axial defects. However, Watanabe and Whitman also concluded that in *Xenopus*, Fast-1 was essential for the expression of many mesoendodermal genes (Watanabe and Whitman, 1999), whereas we found only modest effects with the morpholino approach. This apparent contradiction is easily reconciled. Overexpressed FAST-En^{R} will bind any promoter with a Fast-binding site and will actively repress that gene, even if the XFast normally only contributes partially to its transcriptional activation. By contrast, when levels of XFasts are knocked down, large effects will only be observed on target genes for which ARF complexes are the predominant activators. Thus, genes activated by ARFs in combination with other transcriptional activators will be completely repressed by the dominant-negative approach, but not necessarily by the knockdown approach. A dramatic demonstration of the different outcomes of these two approaches is seen by comparing the phenotype of the zebrafish Fast-1 null mutant, with the much more severe phenotype of zebrafish embryos overexpressing dominant negative Fast-1 (Stemple, 2000).

XFast-1 and XFast-3 in nodal/derrière signalling

The phenotypes observed in the mouse and zebrafish resulting from the loss of Fast-1, and now in *Xenopus* resulting from the knockdown of XFast-1 and XFast-3 levels, are reminiscent of the phenotypes observed in embryos in which nodal signalling is lost, but are much less severe (Whitman, 2001). This suggests that the Fasts are not the only transcription factors capable of recruiting active Smad complexes to target promoters in response to nodal/derrière signalling. Other transcription factors known to play this role are the *Xenopus* homeodomain proteins Mixer, Milk and Bix3 and the Mixer orthologue in zebrafish, bonnie and clyde (Germain et al., 2000; Randall et al., 2002); there are also likely to be others (Howell and Hill, 1997).

The major role of ARF1 and ARF2 in *Xenopus* is in regulating gastrulation movements

Our data suggest a major role for XFast-1/ARF1 and XFast-3/ARF2 in re-programming cell polarity and cell-cell interactions necessary for the convergent extension movements of gastrulation in response to activin-related signals. Indeed XFast-1 and XFast-3 are spatially and temporally well placed to perform this role. At least some of the targets of ARF1 and ARF2 are therefore likely to be genes required for changing cell polarity and adhesion.

In addition to the activin-related signalling pathway, two other signalling pathways have been implicated in gastrulation movements in the *Xenopus* embryo. These are a non-canonical Wnt pathway activated by XWnt11 (Djiane et al., 2000; Heisenberg et al., 2000; Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000), and an FGF-dependent signal transduction pathway that is sensitive to XSprouty2 (Nutt et al., 2001). In both cases, inhibiting these pathways has only limited effects on mesoendodermal gene induction, but greatly inhibits activin-induced elongation of animal caps, indicating that signalling through these pathways is required for activin-mediated gastrulation movements (Nutt et al., 2001; Tada and Smith, 2000). These pathways may act sequentially, although we have demonstrated that *XWnt11* is not itself a target gene for ARF1 or ARF2, as its expression is unaffected in ARF-depleted embryos. Alternatively, there could be a parallel pathway activated by activin-related ligands, requiring ARF1 and ARF2, that is essential for mediating the convergent extension movements of gastrulation, which synergises with Wnt- and FGF-dependent pathways. Given the involvement of multiple signalling pathways in regulating gastrulation movements, we cannot predict whether the phentotype of ARF-depleted embryos results from large changes in a few key ARF target genes, or small changes in a large number of ARF target genes whose promoters integrate signals from multiple pathways. We are currently screening for ARF target genes to resolve this important issue.

We thank Les Dale, Michael King, Jon Pines, Hazel Sive, Jim Smith, Peter ten Dijke and Malcolm Whitman for providing reagents. We acknowledge NHPP for human recombinant activin A [lot 15365-36(1)] and are very grateful to Mariann Bienz, Mike Jones, Patrick Lemaire, Julian Lewis, Helen McNeill, Jim Smith, Derek Stemple, Richard Treisman and members of the laboratory for comments on the manuscript and stimulating discussions. The work was supported by the Imperial Cancer Research Fund (now Cancer Research UK).

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