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# Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development

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## SUMMARY

**We have examined the role of MAP kinase during mesoderm induction and axial patterning in *Xenopus* embryos. MAP Kinase Phosphatase (MKP-1) was used to inactivate endogenous MAP kinase and was found to prevent the induction of early and late mesodermal markers by both FGF and activin. In whole embryos, MKP-1 was found to disrupt posterior axial patterning, generating a phenotype similar to that obtained with a dominant inhibitory FGF receptor. Overexpression of either constitutively active MAP kinase or constitutively active MAP kinase (MEK) was sufficient to induce Xbra expression, while only constitutively active MEK was able to significantly induce expression of muscle actin. When MAP kinase phosphorylation was used as a sensitive marker of FGF receptor activity in vivo, this activity was found to persist at a low and relatively uniform level throughout blastula stage embryos. The finding that a low level of MAP kinase phosphorylation exists in unstimulated animal caps and is absent in caps overexpressing a dominant inhibitory FGF receptor provides a basis for our previous observation that overexpression of this receptor inhibits activin induction. These results indicate that FGF-dependent MAP kinase activity plays a critical role in establishing the responsiveness of embryonic tissues to mesoderm inducers.**

Key words: activin, MAP kinase, mesoderm induction, signal transduction, *Xenopus*

## INTRODUCTION

Mesoderm is induced in the marginal zone of *Xenopus* blastulae by an inductive signal originating in the underlying vegetal pole cells. Nieuwkoop and colleagues demonstrated that the prospective ectoderm of the animal pole region is also competent to respond to these signals when the marginal zone is removed and animal cap cells are cultured in contact with vegetal pole cells (Nieuwkoop and Faber, 1967). This animal cap assay has since been used to identify a number of secreted factors that are capable of mimicking this inductive process. These factors include members of the fibroblast growth factor such as e-FGF and b-FGF (Kimelman et al., 1988; Isaacs et al., 1992; Slack et al., 1988); and multiple members of the TGF-β superfamily including activin (Smith, 1987), Vg-1 (Dale et al., 1993; Thomsen and Melton, 1993), and several bone morphogenic proteins (BMPs) (Dale et al., 1992; Jones et al., 1992; Koster et al., 1991).

It would appear from the number of molecules identified in early *Xenopus* embryos that can induce mesoderm in an animal cap assay that the induction and patterning of endogenous mesoderm is likely to involve a complex interplay between multiple inducing signals. This was highlighted recently when it was found that induction of mesoderm by activin (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994) and Vg-1 (Schultemerker et al., 1994) requires FGF-mediated signals. The point in the activin/Vg-1 signal transduction pathway at which an FGF signal is required, however, remains to be elucidated.

As mesoderm-inducing factors (MIFs) induce an overlapping set of mesodermal markers, it will be difficult to distinguish the contributions individual factors make to this process in vivo. This task is further complicated by the fact that the earliest transcriptional responses to MIFs are not detectable for 30-60 minutes following the application of growth factors to animal caps (Rosa, 1989; Smith et al., 1991). This makes it difficult to determine whether the induction of a particular gene is in direct response to a given factor, or whether it requires secondary interactions with additional inducing factors and their signaling pathways.

One method that has been used to dissect the contribution of individual MIFs to the mesoderm induction process is the overexpression of dominant interfering receptors. Cytoplasmic deletions of the FGF receptor (Amaya et al., 1991), a tyrosine kinase, and the type IIB activin receptor (Hemmati-Brivanlou and Melton, 1992), a serine/threonine kinase, have been generated and shown to inhibit mesoderm induction by FGF and activin, respectively. Interpretation of these experiments is complicated, however, by the observation that the dominant inhibitory activin receptor can inhibit mesoderm induction by other TGF-β family members (Schultemerker et al., 1994), as well as by the requirement for FGF during mesoderm induction by activin-like inducers (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

An alternative approach to understanding the interaction between different MIFs during mesoderm induction is to identify early pretranscriptional responses that are unique to individual factors or families of factors. We and others have shown that a number of the components of the FGF signal transduction pathway characterized in somatic cells are important for FGF signaling in the early embryo (LaBonne and Whitman, 1994; MacNicol, 1993; Whitman and Melton, 1992). Activation of the FGF receptor appears to activate the small GTPase $p21^{ras}$ (Cai et al., 1990; Thomas et al., 1992; Wood et al., 1992), which in turn activates the cytosolic kinases raf-1 (Dent et al., 1992; Howe et al., 1992) and MAP kinase (reviewed in Blenis, 1993). As with the dominant inhibitory FGF receptor, dominant inhibitory mutants of raf and ras inhibit mesoderm induction by both FGF and activin (LaBonne and Whitman, 1994; Whitman and Melton, 1992). Despite the necessity of a functional FGF signaling pathway for activin induction, activin itself does not cause a significant activation of MAP kinase or $p21^{ras}$ (Graves et al., 1994; LaBonne and Whitman, 1994), indicating that activin does not directly stimulate this pathway. This suggests that early signaling responses can be used to distinguish these pathways in vivo, and thus help define the roles of individual factors or families.

As MAP kinase activation is rapid (within 5 minutes), sustained and easily measured (Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994), MAP kinase appeared to be a good candidate marker for examining FGF activity in vivo. *Xenopus* MAP kinase has been cloned and is expressed throughout early development (Zaitsevskaya and Cooper, 1992). Using a MAP kinase-specific phosphatase (Sun et al., 1993), we demonstrate that MAP kinase activation is required for mesoderm induction by FGF and activin. We also show that activation of MAP kinase is itself sufficient to induce mesodermal markers. We find, however, that during the time of mesoderm induction, endogenous MAP kinase does not appear to be stimulated to levels sufficient for mesoderm induction, suggesting that FGF signaling does not play an instructive role in this process. Instead, MAP kinase activation persists at a constant but low level that is FGF dependent and required for mesoderm induction by activin.

## MATERIALS AND METHODS

### Plasmids and in vitro transcriptions

Constitutively active ras (p21v-Ha-ras) and constitutively active raf have been described elsewhere (LaBonne and Whitman, 1994). Wild-type and constitutively active MEK were a kind gift of Natalie Ahn. *Drosophila* $rl^{sem}$ was kindly provided by Larry Zipursky and Ernst Hafen. ERK1 carrying the $rl^{sem}$ mutation was a kind gift of Karen Schinkmann and John Blenis. MKP-1 and phosphatase dead MKP-1 were kindly provided by Lester Lau and Nick Tonks. cDNAs were subcloned into transcription vectors and transcribed with SP6 or T7 RNA polymerase as previously described.

### Microinjection and animal cap assays

Embryos were collected from *Xenopus laevis* females and fertilized as previously described (Newport and Kirschner, 1982). Fertilized eggs were placed in 3% Ficoll/1.0× MMR for injection, then transferred to 0.1× MMR. Unless otherwise noted, injection was carried out at the 2-cell stage into the animal pole of both blastomeres with 10-15 nl of synthetic mRNA. Concentrations of injected mRNA were as noted in the text. Animal caps were dissected at blastula stages in 1.0× MMR and incubated with 50 ng/ml human recombinant FGF (Gibco/BRL) or 0.5× PIF (provided by S. Sokol) unless otherwise noted. Animal caps were harvested at stage 10.5 for Xbra analysis or after 36 hours for muscle actin analysis unless otherwise noted. Staging of embryos was done according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For cycloheximide (CHX) experiments, animal caps were explanted at stage 8 and pretreated with 5 μg/ml CHX for 30 minutes, treated with activin + 5 μg/ml CHX for 30 minutes followed by a 1.5 hour incubation in activin alone before harvesting for northern blot analysis.

### Northern blots and RT-PCR analysis

Total RNA was isolated from whole embryos and animal caps as previously described (Krieg and Melton, 1987). Northern blots and first strand cDNA synthesis were carried out as previously described (LaBonne and Whitman, 1994). PCR reactions were performed in a 25 μl reaction volume in the presence of trace amounts of $^{32}$P[αdATP] using an annealing temperature of 55°C and 22-25 cycles. For Xbra overexpression experiments, injected mRNA was synthesized from a brachyury cDNA lacking 3′ untranslated sequences, while primers used to detect endogenous brachyury inclued one primer in this region. Primer pairs utilized for RT-PCR were as follows:

Xbra: 5′GGATCGTTATCACCTCTG3′, 5′GTGTAGTCTGTAGCAGCA3′

EF1α: 5′CAGATTGGTGCTGGATATGC3′, 5′ACTGCCTTGATGACTCCTAG3′

Muscle Actin: 5′GCTGACAGAATGCAGAAG3′, 5′TTGCTTGGAGGAGTGTGT3′

### Immunoprecipitations and western blot analysis

Antiphosphotyrosine blots were carried out as previously described (LaBonne and Whitman, 1994). IP-westerns were carried out as follows: animal caps were lysed as previously described (LaBonne and Whitman, 1994), and immunoprecipitated for 3-4 hours at 4°C with antiphosphotyrosine (4G10) coupled protein A sepharose, electrophoresed on 10% polyacrylamide gels and blotted to nitrocellulose. Blots were probed overnight at 4°C with MAP kinase antibody (gift of J. Blenis). Following incubation for 1 hour at room temperature with HRP-conjugated 2° antibody (Jackson Labs), blots were visualized using ECL (Dupont NEN). Samples were standardized to total MAP kinase protein in the lysates and results are reported as percent MAP kinase phosphorylation. For quantitative analysis, IP-westerns or direct westerns of cell lysates were carried out with anti-MAP kinase antibody as above, but secondary antibody was AP-conjugated and development was carried out using Attophos (JBL) fluorescent substrate. Levels of anti-phosphotyrosine-precipitated or total MAP kinase were then quantitated on a Molecular Dynamics Fluorimager using ImagequaNT software.

For estimation of the percentage of total MAP kinase that is tyrosine phosphorylated, uninjected or MKP-1 injected animal caps were treated with 50 ng/ml bFGF or control buffer for 5′, lysed in immunoprecipitation buffer and cleared by centfrifugation for 5 minutes at 16,000 ***g***. A portion of each extract was then analyzed directly by Laemmli gel electrophoresis/anti-MAP kinase western blotting; the remainder was precipitated with anti-phosphotyrosine. Treatment of animal cap tissue with FGF causes approximately half the immuno-detectable MAP kinase to migrate with a slightly reduced mobility; which has been shown to be associated with tyrosine phosphorylation of this protein (Wood et al., 1992). The exact proportion of MAP kinase that undergoes this mobility shift after FGF treatment was determined by fluorimager quantitation of the slower and faster migrating bands of MAP kinase on an anti-MAP kinase western blot of extract from FGF-treated caps, providing the % MAP kinase phosphorylation in FGF-treated caps. To determine the % MAP kinase phosphorylation in untreated caps, the amount of MAP kinase pre-

cipitated by anti-phosphotyrosine from FGF and untreated or MKP-1 treated caps was compared by quantitative fluorimager analysis of anti-MAP kinase western blots of anti-phosphotyrosine immunoprecipitated MAP kinase. Comparison to mobility-shifted MAP kinase in FGF-treated caps, rather than simply determining the ratio of anti-phosphotyrosine immunoprecipitated MAP kinase to total MAP kinase, was necessary because anti-phosphotyrosine immunoprecipitation does not efficiently clear tyrosine phosphorylated protein from lysates.

### RSK kinase assays

10-15 animal caps were lysed in 20 μl of lysis buffer containing 50 mM Hepes, 5 mM EDTA, 2 mM Vanadate, 1 mM Molybdate, 20 mM NaF, 10 mM β-glycerol phosphate and protease inhibitors. 3 μl of this lysate was assayed for RSK kinase activity for 10 minutes at RT in a 40 μl reaction containing 50 mM Hepes, 20 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 1 μg recombinant RSK, 50 μM ATP and 0.5 μl $^{32}$P[γATP]. Kinase reactions were resolved on 10% acrylamide gels and quantitated on a Molecular Dynamics phosphoimager.

### Preparation of thio-phosphorylated MAP kinase

Wild-type or kinase dead (K52R) ERK1 overexpressed in and purified from *E. coli* (provided by Dr E. Shibuya) was phosphorylated using 1 mM ATPγS with activated MEK (provided by Dr W. Haser) purified from Sf9 cells coinfected with baculovirus constructs containing hexahistidine-tagged MEK and v-ras. After phosphorylation, MEK was removed with Ni-NTA agarose (Qiagen) and ATPγS removed by repeated washing of the reaction mix on a 30 kD cutoff Microcon (Amicon) filter.

### Histology and immunocytochemistry

Embryos were fixed in 1× MEMFA for 1 hour at room temperature with nutation as previously described (LaBonne and Whitman, 1994). For histology, fixed embryos were dehydrated through an ETOH series and embedded in paraplast. 10 μm sections were dewaxed and stained with Giemsa. Whole-mount immunocytochemistry with 12/101 antibody was as described (Kintner and Brockes, 1984).

### Digital imaging

Autoradiographs of northern blots and RTPCR gels were digitized using a Kodak digital camera, imported into Adobe photoshop for lane labeling and printed on photographic paper. For northern blots, multiple probings of the same filter were overlayed and photographed together to maintain the relative spacing of the bands. For RT-PCR runs, products were resolved on separate gels and photographed together, and these images have been separated to reflect this.

## RESULTS

### Activation of MAP kinase is sufficient for mesoderm induction

In *Xenopus*, it has been previously demonstrated that overexpression of activated mutants of p21$^{ras}$ or raf-1 is sufficient to induce mesoderm in an animal cap assay (LaBonne and Whitman, 1994; MacNicol et al., 1993; Whitman and Melton, 1992). As with FGF induction, activation of p21$^{ras}$ or raf-1 causes a strong phosphorylation and activation of MAP kinase (LaBonne and Whitman, 1994). raf-1 is one member of a family of kinases that serine phosphorylate and activate MAP kinase kinase (MEK) (Kyriakis et al., 1992), which in turn phosphorylates and activates MAP kinase (Mitogen Activated Protein Kinase) also called ERK (Extracellularly Regulated Kinase). Although p21$^{ras}$ and raf-1 were found to activate MAP kinase, presumably in a MEK-dependent fashion, it was not clear whether this activation was either necessary or sufficient for transduction of the mesoderm-inducing signal by upstream components of this pathway. Although in somatic cells constitutive activation of MEK has biological effects very similar to those of activation of p21$^{ras}$ and raf-1 (Mansour et al., 1994; Cowley et al., 1994), whether MAP kinase is the only important target of the ras/raf/MEK transduction pathway is not certain. To address the question of whether MAP kinase activation, like activation of p21$^{ras}$ and raf-1, is itself sufficient to generate a mesoderm-inducing signal, we overexpressed constitutively active mutants of MEK or MAP kinase in early embryos.

One way to activate MAP kinase in early embryos is to upregulate the activity of its upstream activating kinase, MAP kinase kinase (MEK). Several constitutively active MEK mutants have recently been engineered that have basal activities up to 400× greater than that of the wild-type kinase (Mansour et al., 1994). The greatest increase in basal activity is achieved by changing two regulatory serines (ser218, ser222) to aspartic acid and by deleting a region outside the catalytic core that is thought to stabilize the inactive state of the kinase. mRNA coding for a mutant MEK carrying all three of these mutations was injected into *Xenopus* embryos at the 2-cell stage. Animal caps were allowed to develop to stage 10 or stage 18 before being harvested for northern blot analysis. Fig. 1 demonstrates that activation of MEK is sufficient to induce the expression of *Xenopus brachyury* (Xbra), an immediate early mesoderm response gene (Smith et al., 1991), as well as expression of muscle actin, a marker of differentiated mesoderm (Gurdon et al., 1985). When animal caps were analyzed at stage 9 by anti-phosphotyrosine western blot, a significant increase in MAP kinase phosphorylation was observed (not shown). As MAP kinase is the only identified MEK substrate, these results suggest that activation of MAP kinase itself should be sufficient to induce mesodermal markers. In order to test this, we overexpressed a constitutively active MAP kinase mutant.

Recently, a genetic screen in *Drosophila* for dominant mutations that specify R7 photoreceptor cells independent of the receptor tyrosine kinase *sevenless* or its ligand *BOS* resulted in the isolation of *sevenmaker*, a dominant gain-of-function mutation in the *Drosophila* MAP kinase gene *rl* (Brunner et al., 1994). This mutation is a single amino acid substitution (D334N) in a conserved residue in kinase subdomain XI. The biochemistry of how this mutation results in constitutive activation of the kinase is as yet unclear. The *sevenmaker* phenotype mimics a weak gain-of-function mutation in sevenless, suggesting that either MAP kinase is only partially capable of transducing the signal from this receptor, or that the D334N mutation results in only a partial activation of MAP kinase. Overexpression studies in *Drosophila* seem to support the latter hypothesis.

To assay the ability of an activated MAP kinase to induce mesoderm, mRNA coding for the *Drosophila rl*$^{sem}$ mutant (provided by Drs L. Zipusky and E. Hafen), or for p44MAP kinase (ERK1) carrying the corresponding mutation (ERK$^{sem}$) (provided by Drs K. Schinkmann and J. Blenis) was injected into the animal pole of *Xenopus* embryos at the 2-cell stage. Animal caps were explanted at stage 8.5 and allowed to develop to stage 10 or 18 before harvesting for northern blot analysis. Fig. 1A demonstrates that both activated MAP

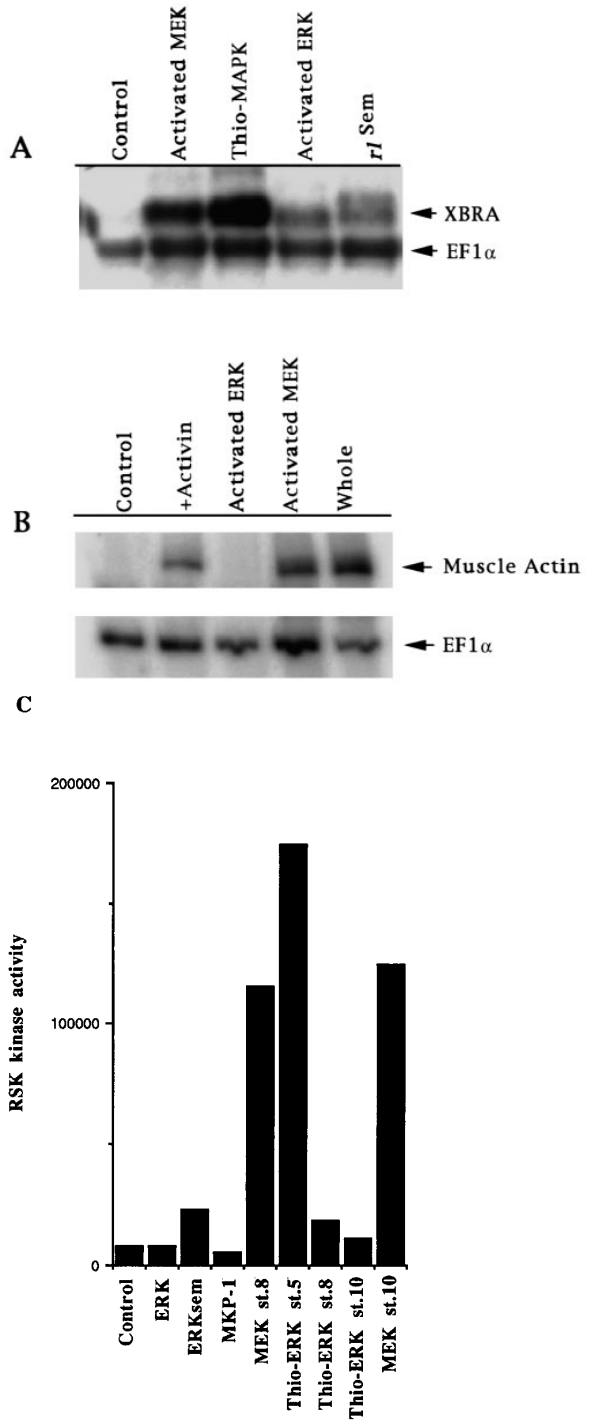


**Fig. 1.** Induction of mesoderm by activated MEK and MAP kinase. (A) mRNA encoding mutationally activated MEK, ERK or *Drosophila* ERK ($rl^{sem}$) or thiophosphorylated MAP kinase protein was injected at stage 2; animal caps were cut at stage 8.5, RNA harvested at stage 10 and analyzed by northern blot probed for Xbra. Blot was reprobed with EF1α as a loading control. (B) Activated ERK or MEK RNA was injected at stage 2 and animal caps were cut at stage 8.5. RNA was harvested at stage 18 and analyzed by RT-PCR using primers specific for muscle actin or EF1α. Kinase dead thiophosphorylated MAP kinase protein or ERK RNA did not induce Xbra (not shown). (C) Animal caps injected with activated MEK, $ERK^{sem}$ or thiophosphorylated ERK were explanted at stage 5, 8 or 10 and crude whole cell lysates were analyzed for RSK kinase activity.

kinases were able to induce Xbra expression in this assay, although this activation is somewhat weaker than can be achieved with FGF, activated $p21^{ras}$ or activated raf-1. Overexpression of these activated MAP kinase mutants also caused a weak elongation of animal caps (not shown). The activity of the $rl^{sem}$ mutant was indistinguishable from vertebrate MAP kinase in these assays, demonstrating that MAP kinase function and specificity are highly conserved across metazoan development. Fig. 1B demonstrates that overexpression of the activated MAP kinase mutant was not sufficient to induce a marker of differentiated mesoderm, muscle actin. This could be explained if D334N results in only a weak activation of MAP kinase. Alternatively, activation of MAP kinase may not be sufficient to transduce the mesoderm-inducing signal in its entirety, allowing initiation but not maintenance of the mesoderm response.

An alternative way to activate MAP kinase in early embryos is to inject thio-phosphorylated MAP kinase protein (Haccard et al., 1993). Highly activated MAP kinase protein was generated in vitro and injected into embryos at the 2-cell stage. This protein was phosphorylated with γ-S-ATP to render it resistant to dephosphorylation by protein phosphatases. As has been previously described, high concentrations of this preparation resulted in cleavage arrest of injected blastomeres (Haccard et al., 1993). Animal caps were explanted from embryos expressing lower doses of this protein and were analyzed for the expression of mesodermal markers. As was found for MAP kinase carrying the D334N mutation($ERK^{sem}$), thio-phosphorylated MAP kinase protein induced the early response gene Xbra, but did not significantly induce muscle actin (Fig. 1A,B and not shown).

Of the three methods used to activate MAP kinase activity, only the overexpression of activated MEK was able to induce muscle actin expression. This suggests either that MEK has physiologically important substrates other than MAP kinase, or that MEK elevates endogenous MAP kinase activity beyond the levels achieved by either $ERK^{sem}$ or the thio-phosphorylated protein, and that this elevation is required for induction of markers of differentiated mesoderm by MAP kinase. MEK substrates other than MAP kinase have yet to be identified in other systems. To compare the relative amounts of MAP kinase activity generated by the three methods of MAP kinase activation described above, whole cell extracts were prepared from animal caps expressing activated ERK, activated MEK or thio-phosphorylated MAP kinase protein, and were assayed for their ability to phosphorylate $pp90^{RSK}$ (ribosomal S6 kinase) (Fig. 1C). $pp90^{RSK}$ is a physiological substrate of MAP kinase (Blenis, 1993), and MAP kinase is the predominant source of RSK kinase activity in a whole cell lysate. Because of this, RSK phosphorylation is a useful marker for MAP kinase activity. Fig. 1C demonstrates that animal caps expressing $ERK^{sem}$ have 3-fold greater RSK kinase activity than uninjected animal caps. This is the first observation of increased kinase activity for a MAP kinase carrying this mutation. Animal caps overexpressing the activated MEK displayed a 15-fold increase in RSK kinase activity relative to controls. The difference in the abilities of activated MEK and activated ERK to increase MAP kinase activity may well explain their differing abilities to induce late markers of mesoderm induction.

As has been previously demonstrated, embryos injected with

thio-phosphorylated MAP kinase have high levels of MAP kinase activity at early cleavage stages (Haccard et al., 1993). By stage 8, however, these levels are only slightly higher than those obtained by overexpressing the $ERK^{sem}$ mutant and, by gastrula stages, these levels approach background (Fig. 1C). This decrease in activity probably reflects degradation of the injected protein. We cannot conclude from this result that MAP kinase activation is insufficient for induction of late mesodermal markers, as high levels of activity are not maintained through these stages. If, as suggested by the ability of MEK to induce muscle actin, activation of MAP kinase is insufficient for induction of differentiated mesoderm, the results obtained with this protein would suggest that initiation of the mesoderm response is not sufficient to maintain this fate in the absence of a continued MAP kinase signal.

### MAP kinase activation is required for mesoderm induction by RAS and RAF

MKP-1 is a dual specificity (threonine/tyrosine) phosphatase that has been shown to dephosphorylate and inactivate MAP kinase in vivo with high specificity. In somatic cells expressing MKP-1, tyrosine phosphorylation of MAP kinase is inhibited while other tyrosine phosphoproteins are unaffected (Sun et al., 1993). Recently it has been demonstrated that MKP-1 fails to inactivate kinases closely related to MAP kinase such as JNK-1 (Sun et al., 1994). To establish that mesoderm induction by upstream components of the FGF signaling pathway requires MAP kinase activation, RNA coding for MKP-1 was co-injected with RNA coding for constitutively active forms of $p21^{ras}$ or raf-1. Animal caps were explanted at stage 8.5 and harvested at stage 10 for northern blot analysis. While expression of 100 pg of either $p21^{v\text{-}ras}$ or activated raf-1 alone was sufficient to induce expression of Xbra, an immediate early mesoderm response gene, co-expression of MKP-1 eliminated this induction (Fig. 2A). MKP-1 expression had no effect on expression of a housekeeping gene, EF1-α, suggesting that this inhibition is mesoderm specific. When whole cell extracts from animal caps expressing constitutively active $p21^{ras}$ or raf-1 in the presence or absence of MKP-1 were analyzed by anti-phosphotyrosine western blot, loss of $p21^{ras}$ or raf-1-mediated mesoderm induction was found to correlate with a loss of MAP kinase phosphorylation in MKP-1 expressing cells (Fig. 2B,C). As has been previously described (Sun et al., 1993), MKP-1 did not diminish tyrosine phosphorylation of proteins other than MAP kinase, and did not prevent prevent phosphorylation of $p70^{rsk}$ (Fig. 2B and not shown). These results demonstrate that MAP kinase activation is required for mesoderm induction by $p21^{ras}$ and raf-1, and suggest that it may be necessary for induction by MIFs that require a ras-mediated FGF signaling pathway.

### MAP kinase activation is required for mesoderm induction by FGF, activin and Brachyury

To establish whether MAP kinase activation is required for mesoderm induction by peptide growth factors, animal caps from embryos injected with 2 ng of MKP-1 mRNA at the 2-cell stage were explanted at stage 8.5 and treated with FGF or activin. Untreated animal caps expressing MKP-1 differentiated as atypical epidermis indistinguishable from uninjected control caps. These caps continued to express epidermal keritin and did not express β-tubulin, a marker of neural induction. Uninjected caps treated with FGF or activin underwent characteristic extension movements thought to mimic the convergence and extension of endogenous mesoderm during gastrulation and neurulation (Symes and Smith, 1987). In animal caps expressing MKP-1, these movements were severely inhibited in response to both FGF and activin (Fig. 3A). Animal caps were harvested at stage 10 and stage 36 to

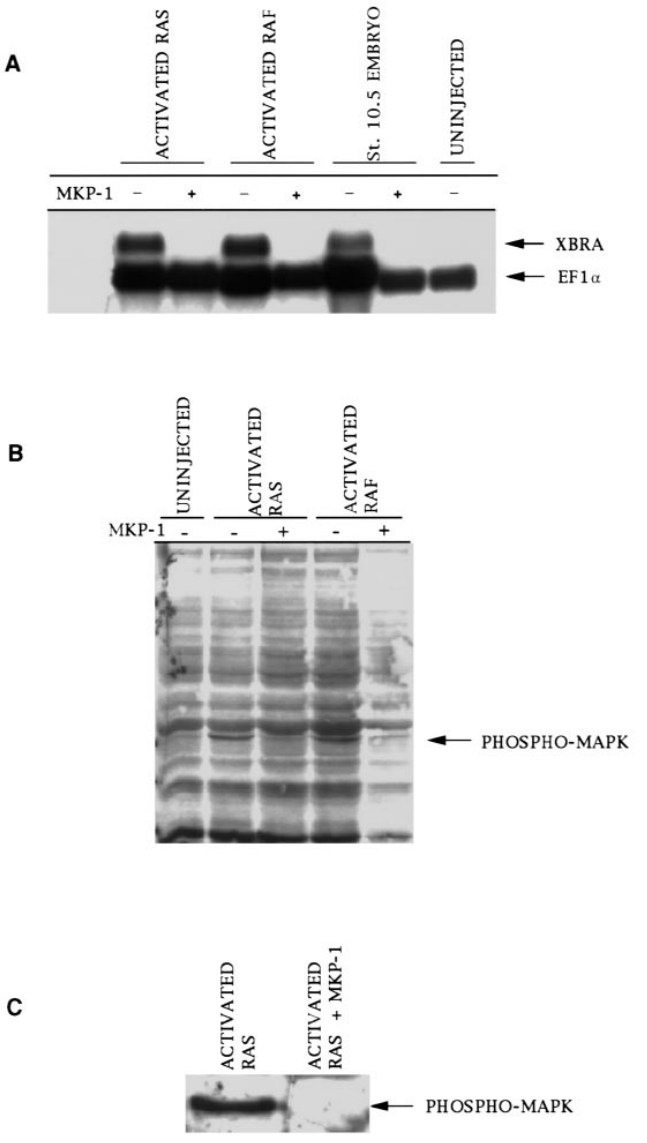


**Fig. 2.** Inhibition of brachyury induction by MAP kinase phosphatase (MKP-1). Embryos were injected in both cells at the 2-cell stage with RNA encoding activated ras, activated raf and/or MKP-1 RNA, animal caps were cut at stage 8.5 and harvested for RNA or protein at stage 10. (A) Effect of MKP-1 on induction of Xbra in activated ras or activated raf injected embryos, and in intact uninjected embryos. Northern blots were probed for the mesoderm immediate response gene brachyury and EF1α. (B) Anti-phosphotyrosine western blot demonstrating the effect of MKP-1 on tyrosine phosphorylation of MAP kinase in activated ras or activated raf injected embryos. (C) Phosphotyrosine-containing proteins were immunoprecipitated from animal caps overexpressing $p21^{ras}$ +/– MKP-1 and analyzed by western blot for MAP kinase levels. MKP-1 eliminates all tyrosine phosphorylated MAP kinase.

examine the effects of MKP-1 on induction of early and late mesodermal markers. MKP-1 inhibited the induction of the immediate early gene Xbra in response to both FGF and activin (Fig. 3B). Similarly, induction of muscle actin, a marker of differentiated mesoderm, was inhibited in response to both inducing factors (Fig. 3C). MKP-1 appears to be equally effective at inhibiting mesoderm induction by both factors at all doses examined (not shown). It has been previously demonstrated that FGF but not activin induces a strong and rapid phosphorylation of MAP kinase in animal pole blastomeres. The requirement for MAP kinase during activin-mediated mesoderm induction does not therefore indicate that activin signals directly through a MAP kinase cascade. Instead, as has been suggested for $p21^{ras}$ and raf-1, MAP kinase appears to be a necessary step in an FGF signaling pathway that is itself required for aspects of activin-mediated mesoderm induction (LaBonne and Whitman, 1994).

It appears likely that a functional FGF signaling pathway may be required for mesoderm induction by a wide range of inducers. It has recently been demonstrated that induction of Xbra by Vg-1, another TGF-β family member, is FGF dependent (Schultemerker et al., 1994). It was unclear whether mesoderm induction by molecules unrelated to these two families would also require an FGF signal. Overexpression of the transcription factor brachyury (Xbra) has been found to induce its own expression in animal caps (Rao, 1994), as well as expression of other mesodermal markers such as Xhox3, Xsna, Mix1 and muscle actin (Cunliffe and Smith, 1992; Rao, 1994). We asked whether Xbra could induce mesoderm in animal caps in the absence of a functional FGF signaling pathway. When either MKP-1 or a dominant inhibitory FGF receptor were co-expressed with Xbra, they were found to inhibit Xbra's ability to induce its own expression (Fig. 4A), as well as inhibiting Xbra-mediated elongation of animal caps and

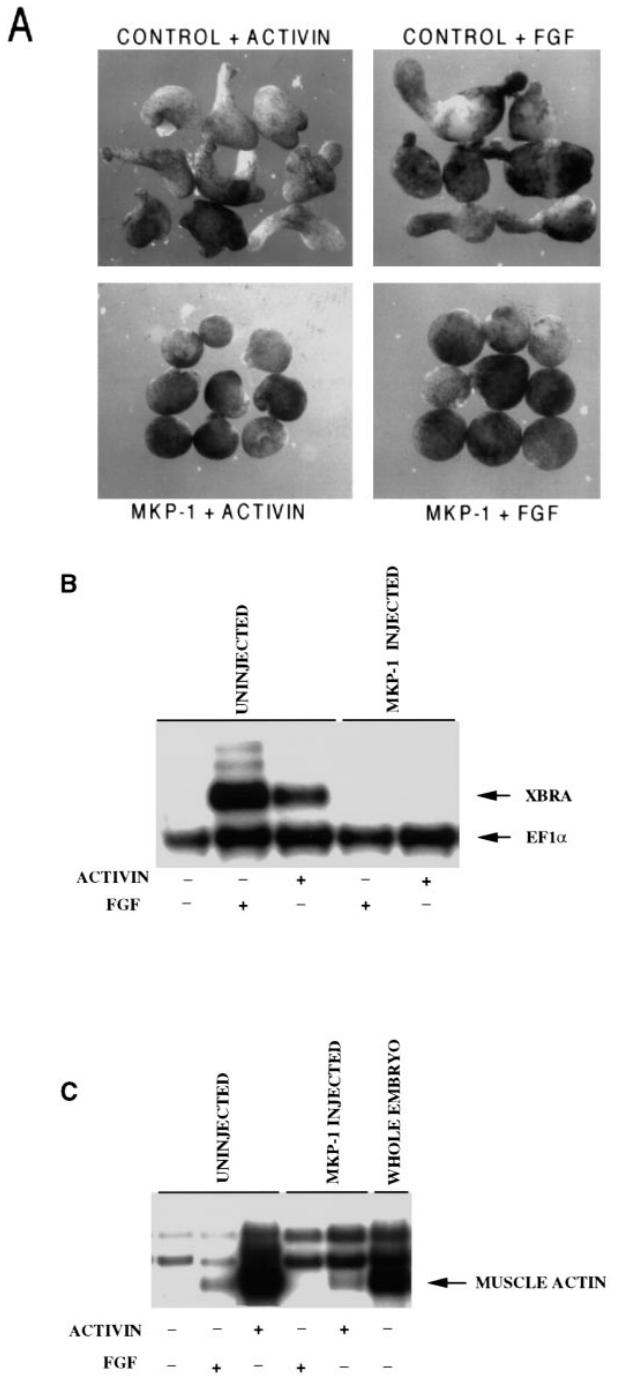


**Fig. 3.** MKP-1 inhibits mesoderm induction by FGF and activin. Embryos were injected in both cells at the 2-cell stage with RNA encoding MKP-1, animal caps were cut at stage 8.5, treated with activin or FGF and (A) photographed for elongation characteristic of mesoderm induction at Stage 13, or harvested at stage 10.5 or stage 36 for northern blot analysis using probes for Xbra, EF1α (B) or muscle actin (C). (C) Cytoskeletal actin (top two bands) serves as an RNA loading control.

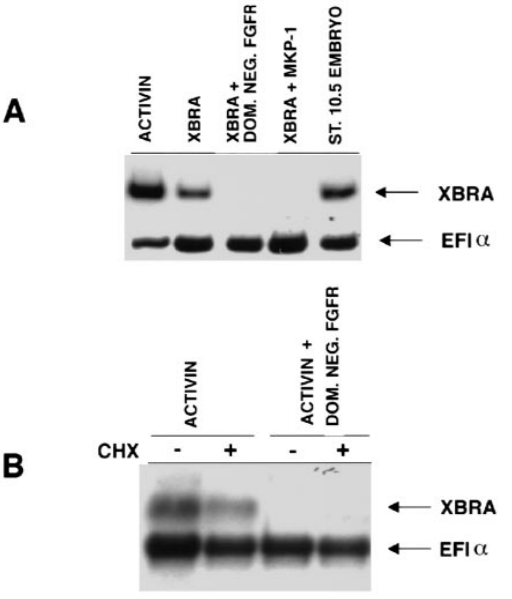


**Fig. 4.** FGF signaling is required for immediate early mesoderm responses. (A) Embryos were injected at the 2-cell stage with RNA encoding Xbrachyury +/– dominant inhibitory FGF receptor or MKP-1. Animal caps were explanted at stage 8.5 and harvested at stage 10.5 for RT-PCR analysis using primers specific for endogenous Xbra or EF1α. (B) Control animal caps or animal caps overexpressing dominant inhibitory FGF receptor were explanted at stage 8 and treated with activin in the presence or absence of CHX as described (Rosa, 1989), then harvested for northern blot analysis using probes specific for Xbra or EF1α.

induction of muscle actin expression (not shown). Although FGF can induce Xbra in animal caps, these results suggest that Xbra induction is insufficient to maintain its own expression in the absence of a continued FGF signal. It also suggests that an FGF signal is required for even the most immediate responses to mesoderm-inducing signals. This was confirmed by treating animal caps with cycloheximide, an inhibitor of protein synthesis. Under these conditions only immediate early responses to activin are observed (Cascio and Gurdon, 1987; Rosa, 1989). Fig. 4B demonstrates that blocking the FGF signaling pathway in the presence of cycloheximide is still sufficient to inhibit activin induction of the immediate early gene Xbra, suggesting that a functional FGF signaling pathway is required for the most immediate responses to activin. MAP kinase activation appears to be essential for this process, as MKP-1 can antagonize MAP kinase activation and inhibit mesoderm induction in *Xenopus* animal pole cells. This is consistent with results in other systems where MAP kinase activation has recently been demonstrated to be required for ras-dependent stimulation of DNA synthesis in fibroblasts (Sun et al., 1994) and specification of R7 photoreceptor cells in *Drosophila* (Biggs et al., 1994).

### MKP-1 effects can be rescued by activated MAP kinase

The specificity of MKP-1 for MAP kinase in somatic cells has been well documented (Alessi et al., 1993; Biggs et al., 1994; Charles et al., 1993; Sun et al., 1993, 1994). As in fibroblasts, in *Xenopus* animal pole cells expressing MKP-1, phosphorylation of MAP kinase is specifically lost while phosphorylation of other proteins is unaffected. Our observation that a MAP kinase carrying the $rl^{sem}$ mutation has constitutive activity in *Xenopus* animal pole cells provides an additional tool for evaluating the specificity of MKP-1.

*Xenopus* embryos were injected at the 2-cell stage with mRNA coding for MKP-1 alone or co-injected with mRNA for the activated MAP kinase mutant at concentrations (0.4 ng) insufficient for mesoderm induction. Animal caps were explanted at stage 8.5 and tested for their ability to respond to activin. Animal caps were harvested at stage 10, and RNA extracted for northern blot analysis of Xbra expression. Activin induced Xbra expression in control caps, while caps expressing MKP-1 failed to be induced. Co-expression of the activated MAP kinase was able to rescue the effects of MKP-1 and restored Xbra expression to control levels (Fig. 5A). Elongation of animal caps in response to activin was also rescued (not shown). Similar results were obtained with the *Drosophila* $rl^{sem}$ mutant. Co-expression of a kinase dead MAP kinase did not significantly rescue the effects of the phosphatase(not shown). As a further test of specificity, RNA coding for defective MKP-1, in which the phosphatase active site cysteine has been mutated to serine, was injected into embryos at the 2-cell stage. Animal caps explanted from embryos overexpressing this phosphatase dead MKP-1 retained responsiveness to both activin and FGF (Fig. 5B). These results demonstrate that the effect of MKP-1 on Xbra expression is specific to its ability to inactivate MAP kinase and that restoration of MAP kinase activity can overcome this block.

### MKP-1 expression causes axial deficiencies

When mRNA coding for MKP-1 is injected in the marginal zone of 2-cell embryos, and these embryos are allowed to develop to tadpole stages, normal development is severely perturbed. Injected embryos fail to complete gastrulation and display posterior axial deficiencies reminiscent of those seen in embryos overexpressing a dominant inhibitory FGF receptor (Amaya et al., 1991), dominant inhibitory ras (Whitman and Melton, 1992) or dominant inhibitory raf (MacNicol et al., 1993) (Fig. 6). However, MKP-1 injected embryos display additional anterior deficiencies at a higher frequency than seen with the dominant inhibitory FGF receptor, including loss of eyes. In these embryos, early movements of gastrulation occur normally and on time. At mid to late gastrula stages, normal closure of the blastopore is suspended and to some degree reversed. Because of this, lateral and ventral regions of the IMZ fail to involute. While a comparatively normal head is formed, indicating that involution of the dorsal mesoderm has occurred, trunk structures are absent or severely diminished. Fig. 6B shows MKP-1 embryos stained with the somite-specific antibody 12/101. While somitic organization is observed in control embryos, in embryos overexpressing MKP-1, 12/101 staining was diminished or absent. Similar results have been obtained by examining the expression of muscle-specific actin in embryos expressing MKP-1 (not

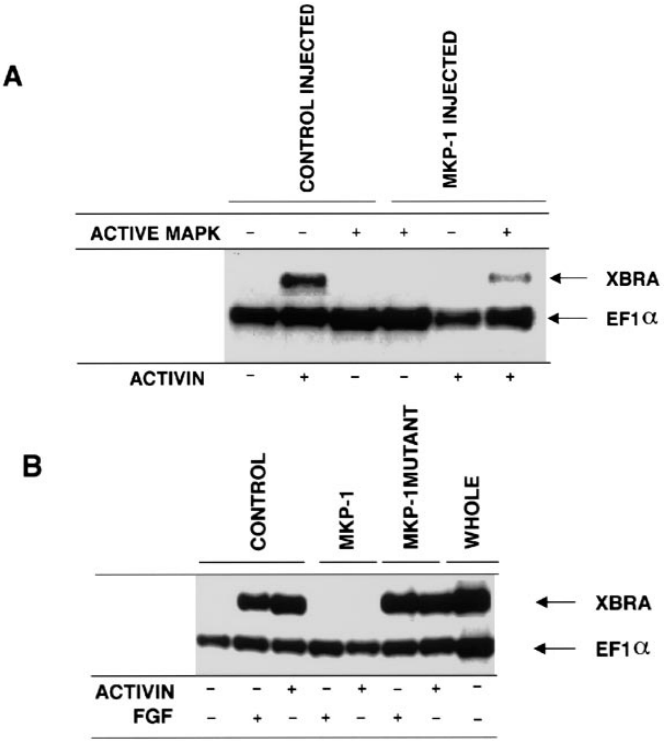


**Fig. 5.** Rescue of MKP-1 inhibition by activated MAP kinase. (A) Embryos were injected with 2 ng RNA encoding MKP-1 alone or co-injected with 0.5 ng RNA encoding activated ERK. Caps were cut and treated with activin at stage 8.5 and harvested at stage 10 for northern blot analysis. Activated MAP kinase RNA is not sufficient to induce brachyury at this dose (0.4 ng) (third lane), but restored the ability of activin to induce brachyury in the presence of MKP-1 (sixth lane). (B) Embryos were injected with 2 ng RNA encoding MKP-1 or phophatase dead MKP-1, caps were explanted at stage 8.5 and treated with FGF or activin and harvested at stage 10.5 for northern analysis. Mutant MKP-1 does not inhibit Xbra induction by FGF or activin.

shown). Histological examination of these embryos reveals that muscle and notochord are greatly diminished and highly disorganized in MKP-1 expressing embryos (Fig. 6C,D). Somitic organization was absent, and notochord, when present, was observed in patches. At lower doses of MKP-1, gastrulation defects can be obtained without significant effects on mesoderm induction, suggesting that these phenomena are separable, and may both be MAP kinase-dependent.

### MAP kinase activation is a marker for FGF activity

As MAP kinase appears to be both necessary and sufficient for some aspects of mesoderm induction and, as its activation can be rapidly assayed, it can be used as a sensitive pretranscriptional marker for FGF activity in vivo. We and others have previously shown that MAP kinase is rapidly (within 30 seconds) phosphorylated and activated in response to FGF but not activin (Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994). These data suggested that although a functional FGF signaling pathway is required for activin induction, FGF is not simply 'downstream' of activin during mesoderm induction. However, alternative models in which a functioning FGF signaling pathway is a prerequisite for competence to respond to activin require that there be a pre-existing FGF signal in unstimulated animal caps. Using MAP kinase phosphorylation as a marker for FGF activity, we looked for such a signal in animal caps during mesoderm induction stages.

Animal caps were explanted from stage 7, 8 and 9 embryos, lysed and phosphotyrosine-containing proteins were immunoprecipitated with the anti-phosphotyrosine antibody 4G10 coupled to protein A sepharose. These immune complexes were resolved by polyacrylamide gel electrophoresis and subjected to western blot analysis with an anti-MAP kinase antibody. This two step procedure was used to maximize the sensitivity of the assay. Fig. 7A demonstrates that phospho-MAP kinase is present in

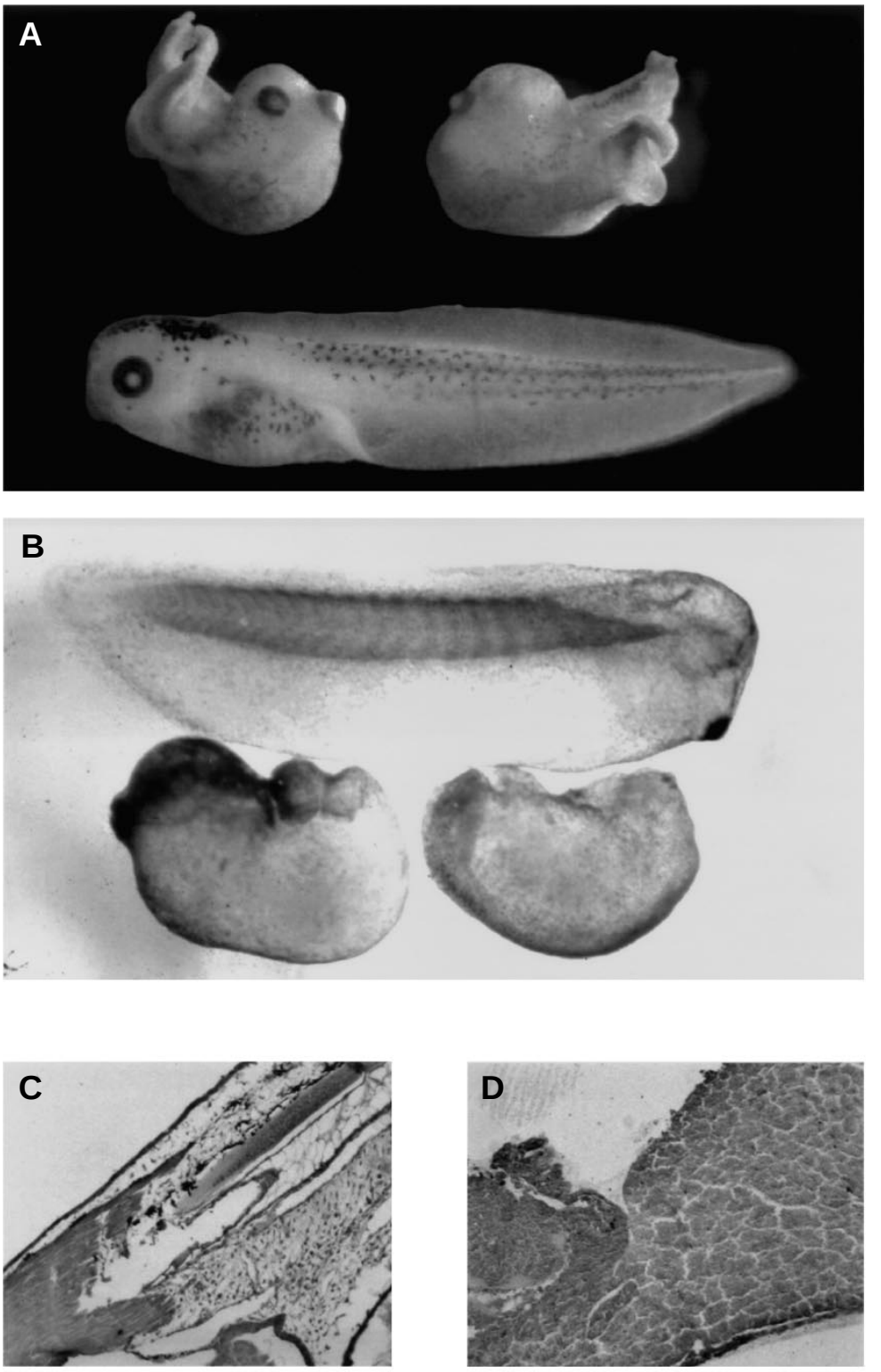


**Fig. 6.** Effect of MKP-1 on embryonic pattern. Embryos were injected in the marginal zone of both cells at the 2-cell stage and (A) photographed at stage 41, (B) were fixed at stage 29-30 for whole-mount immuno cytochemistry and using the antibody 12/101 which recognizes a somite-specific antigen (Kintner and Brockes, 1984) or (C-H) fixed at stage 37/38 for histological analysis. (A) Bottom embryo is control injected, top two are sibling embryos that were injected with 2 ng MKP-1. (B) Bottom two embryos are MKP-1 injected; top embryo is a stage matched control injected embryo. (C) Enlargement of sagittal section of control embryo showing differentiated somite and notochord. (D) Enlargement of sagittal section of MKP-1 injected embryo showing lack of organized somite and notochord.

unstimulated animal caps at low levels. These levels represent approximately 5% of total cellular MAP kinase (Fig. 7C). As a control to confirm that the observed signal represented phosphorylated MAP kinase, immunoprecipitation was carried out in the presence of 2 mM free phosphotyrosine, which competitively inhibits recognition of tyrosine phosphorylated proteins by anti-phosphotyrosine antibody. Under these conditions, no MAP kinase signal is observed (Fig. 7A, lane 1), confirming that the MAP kinase observed by western blot analysis is tyrosine phosphorylated. Overexpression of the MAP kinase phosphatase MKP-1 also reduces tyrosine phosphorylated MAP kinase to undetectable levels without affecting total MAP kinase levels, confirming that MAP kinase precipitation with 4G10 is due to its state of phosphorylation (Fig. 7C). In animal caps expressing a dominant inhibitory FGF receptor or dominant inhibitory ras, this phosphorylation is greatly diminished (Fig. 7A), demonstrating that the observed MAP kinase signal was generated by endogenous FGF activity, or by an FGF-like activity mediated through this signaling pathway.

The presence of a low level FGF signal in unstimulated animal caps explains how a dominant inhibitory FGF receptor can affect mesoderm induction by activin and brachyury. Why this low level signal is required for mesoderm induction remains unclear. The amount of phosphorylated MAP kinase in unstimulated animal caps remains unchanged throughout blastula stages (Fig. 7B,C). Since unstimulated caps differentiate as ciliated epidermis and not mesoderm, this level of activity is clearly below the threshold required for mesoderm induction. Induction of mesoderm by exogenous FGF in an animal cap assay appears, therefore, to be a function of stimulating a pre-existing signal in animal caps to levels significantly greater levels than those seen by these cells during normal development.

We next wished to ascertain how the low level of FGF-like activity present in animal caps compared to what was present in other regions of the embryo during mesoderm induction stages. Animal caps, marginal zones and vegetal bottoms were isolated from early, mid and late blastulae embryos, lysed and analyzed for endogenous levels of phospho-MAP kinase as above. Over the period of time when mesoderm is thought to be induced in the blastula, this level of activity remained constant and relatively uniform throughout prospective ectoderm and mesoderm, while slightly higher levels were consistently observed in the vegetal endoderm (Fig. 7B,D). As the levels of FGF-like activity in animal pole cells is insufficient

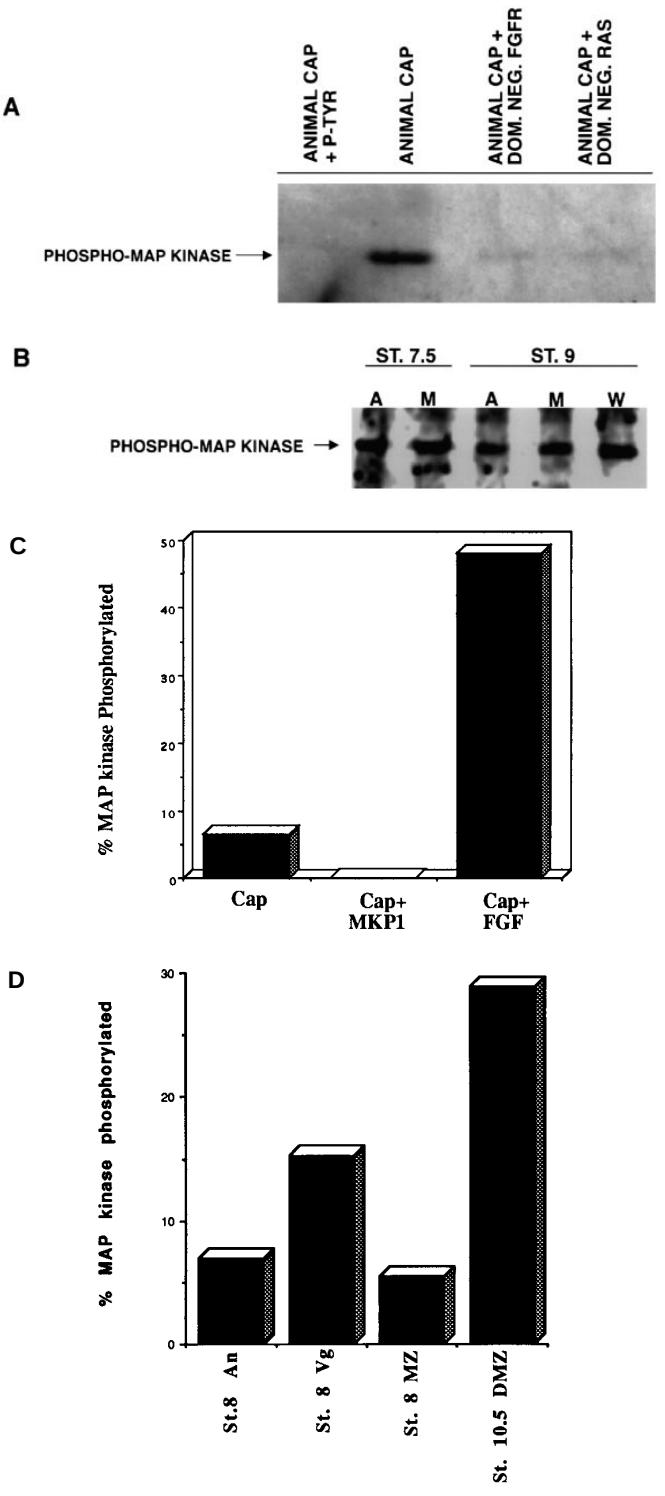


**Fig. 7.** Tyrosine phosphorylation of MAP kinase in embryonic tissues. (A) Embryos injected at the 2-cell stage with RNA encoding dominant inhibitory FGF receptor, dominant inhibitory ras, or water, were cut at stage 8.5, lysed and analysed by anti-phosphotyrosine immunoprecipitation/anti-MAP kinase Western blot. A single band at $44\times10^3$ $M_r$ was observed and quantitated on a fluorescence imager. Total MAP kinase was determined by anti-MAP kinase western blot of a portion of the cell lysates electrophoresed directly (without anti-Ptyr immunoprecipitation) and quantitated on fluorescence imager. (B) Dissected animal caps, marginal zones or whole embryos were lysed at stage 7.5 or stage 9. Lysates were assayed for total MAP kinase content by anti-MAP kinase western blot and standardized for total MAP kinase. Equal volumes were then analysed by anti-phosphotyrosine immunoprecipitation/anti-MAP kinase western blot. A, animal cap; M, marginal zone; W, whole embryo. (C) Uninjected or MKP-1 injected animal caps were cut at stage 8.5 treated with or without 30 ng/ml FGF for 5 minutes and analysed as in B. (D) Embryos were dissected into thirds at stage 8 or stage 10.5 and lysed for anti-MAP kinase Western blot (5% of sample) or anti-phosphotyrosine immunoprecipitation/anti-MAP kinase western blot (95% of sample). Total or phosphotyrosine-MAP kinase was quantitated by fluorimager analysis of western blots; for each sample, the ratio of tyrosine phosphorylated MAP kinase to total MAP kinase was calculated.

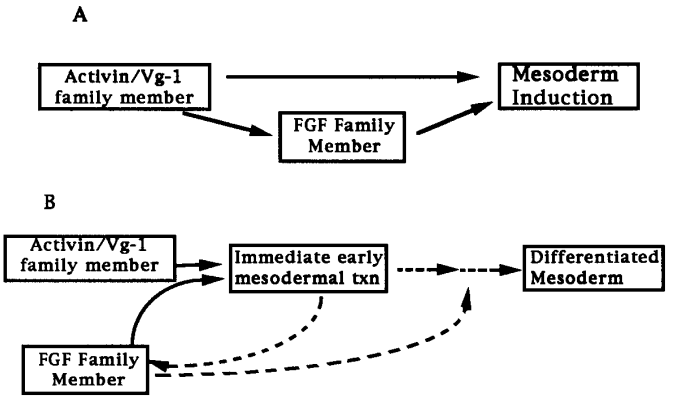


**Fig. 8.** Models for action of FGF in the induction of mesoderm by activin/Vg-1-like factors. Two possibilities for mechanisms underlying the requirement for FGF in the induction of mesoderm by activin/Vg-1. (A) Activin stimulates the production/release of FGF (e.g. eFGF) in target cells, which forms a necessary component of the subsequent inductive response. (B) An FGF signal endogenous to tissues competent to respond to inducer is required in conjunction with an activin/Vg-1 signal for initial mesoderm-specific txn; this initial response includes the production of eFGF, which is then required for the maintenance of the inductive response to the differentiation of mesodermal tissues.

for the specification of mesoderm, and as these levels are the same in the marginal zone as they are in animal caps, the levels of FGF signaling in the marginal zone appears to be too low for FGF to play a primary role in the induction of mesoderm in vivo. Interestingly, phospho-MAP kinase levels increase significantly by stage 10.5, suggesting an additional role for MAP kinase during gastrulation.

## DISCUSSION

A number of intracellular signaling molecules activated by tyrosine kinase receptors in somatic cells have be shown to be important for transduction of the FGF signal in early *Xenopus* embryos. By overexpressing dominant inhibitory mutants, we and others have demonstrated that activation of both $p21^{ras}$ and raf-1 are required for FGF-mediated mesoderm induction. Overexpression of activated mutants of $p21^{ras}$ and raf-1 has also been shown to be sufficient to induce mesoderm in an FGF-like fashion (MacNicol, 1993; Whitman and Melton, 1992). In this paper, we examine the role that a downstream component of this pathway, MAP kinase, plays in transducing the FGF signal.

As has been demonstrated for upstream components of this pathway, overexpression of mutationally activated forms of MAP kinase or MAP kinase kinase (MEK) is sufficient for induction of the immediate early mesoderm response gene Xbra. These two mutants appear to differ, however, in their ability to induce a marker of differentiated mesoderm, muscle actin. While MEK induces muscle actin expression quite well, MAP kinase carrying the $rl^{sem}$ mutation induces this marker poorly if at all. One possible explanation for this follows from the observation that activated MEK elevates endogenous MAP kinase activity to levels significantly (>5 fold) greater than those obtained using the $rl^{sem}$ mutant. It is possible that low level activity is sufficient for initiation of the mesoderm response, but that higher levels are required to sustain the response and allow mesodermal differentiation. The MAP kinase cascade is known to include feedback loops that may amplify its activity. MAP kinase, in addition to phosphorylating downstream substrates also phosphorylates the kinase that activates it, MEK. It is possible that the $rl^{sem}$ mutation does not allow for such signal amplification. Indeed, it is unclear why a conservative change in kinase subdomain XI would lead to activation of the kinase at all. It is possible that rather than being constitutively active itself, this mutant may potentiate pre-existing low levels of endogenous MAP kinase activity. As such a signal is present in *Xenopus* animal caps, this may be the source of the RSK kinase activity that we observe as well. Preliminary evidence suggests that mesoderm induction by the $rl^{sem}$ mutant may itself require phosphorylation by a functional FGF signaling pathway (C. LaBonne, unpublished). If this is indeed the case, the ability of $ERK^{sem}$ to rescue the MKP-1 phenotype may be a stoichiometric rather than a catalytic phenomena.

An alternative explanation for the inability of activated MAP kinase to induce muscle actin is that MAP kinase is insufficient to transduce the entire mesoderm response. As activated MEK does induce markers of differentiated mesoderm, this would imply that MEK has physiological substrates other than MAP kinase. There is at least one observation that suggests such a branch may exist. In *S. pombe*, a mating factor inducible gene has been identified which appears to require the *S. pombe* MEK homologue, *byr2*, but not the MAP kinase homologue, *spk1*, for its induction (Xu et al., 1994). The possibility of such branch points has been suggested as an explanation for why a three-step cascade has been conserved evolutionarily and not replaced by a single kinase.

As it has proven difficult to generate dominant inhibitory forms of MAP kinase that successfully interfere with endogenous MAP kinase activity in early embryos, we have exploited a natural MAP kinase antagonist, MAP kinase phosphatase (MKP-1). Overexpression of MKP-1 in blastula animal caps prevents ras and raf-mediated mesoderm induction, indicating that MAP kinase activation is required for this process. Similarly, we have found that MAP kinase activation is necessary for mesoderm induction by FGF and activin. The requirement for MAP kinase in activin-mediated mesoderm induction does not imply that activin directly activates MAP kinase. Indeed, several groups have found that it does not (Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994). Instead, as has been found previously using dominant inhibitory mutants of the FGF receptor, $p21^{ras}$ or raf-1, MKP-1 inhibition of activin-mediated mesoderm induction reflects a requirement for FGF signaling during this process.

The finding that brachyury-mediated mesoderm induction is itself dependent upon FGF signaling suggests that this pathway is also required for maintenance of the mesoderm response. Xbra may require post-translational modification that is FGF dependent. It is interesting to note that Xbra contains multiple consensus MAP kinase phosphorylation sites in its C terminus. We are currently investigating whether phosphorylation of Xbra by MAP kinase is required for its mesoderm-inducing activity. Alternatively, the ability of overexpressed brachyury to induce eFGF (Isaacs et al., 1994) raises the possibility that

it is this induced eFGF that mediates the dominant negative FGF receptor-inhibited step in induction of mesoderm by brachyury.

Fig. 8 depicts two models for how this requirement might be manifested. In the first model, activation of FGF signaling occurs downstream of activin induction and represents one branch of the activin response. Such a model would suggest that activation of components of the FGF signaling cascade should be measurable following treatment with activin and prior to the induction of the immediate early response. Activation of neither MAP kinase nor $p21^{ras}$ has been observed in response to activin during this period, arguing against this model. The second model depicts FGF signaling as a parallel pathway, which is required both for competence to respond to activin and for maintenance of the activin response. This model requires the presence of low level FGF activity in unstimulated animal caps, and we have found evidence for such a signal as measured by FGFR-dependent MAP kinase phosphorylation. In addition, Isaacs et al. (1994) have shown that eFGF transcription is stimulated by brachyury, an early response gene for mesoderm induction, and that this eFGF expression may be important for mesodermal differentiation. This suggests that, in addition to a role for FGF derived from maternal RNA and protein stores, eFGF expressed zygotically as an early response to induction has a role in maintaining mesodermal specification (Isaacs et al., 1994). Expression of e-FGF has been found to increase in the dorsal marginal zone by the onset of gastrulation (Isaacs et al., 1994), raising the possibility that the increase in MAP kinase phosphorylation that we observe at gastrula stages may be e-FGF mediated.

The requirement for FGF signaling during activin-mediated mesoderm induction does not itself preclude an additional, more direct role for FGF during mesoderm induction. Results obtained with a dominant inhibitory activin receptor suggested, however, that FGF does not play such a role. Animal caps overexpressing a dominant inhibitory activin receptor were found to retain their responsiveness to FGF, while whole embryos overexpressing this dominant inhibitory receptor contain no mesoderm (Hemmati-Brivanlou and Melton, 1992). This strongly suggested that endogenous FGF signaling levels were insufficient for mesoderm induction. Our finding that endogenous levels of MAP kinase stimulation are equivalent in animal caps and marginal zones during mesoderm induction stages, together with the observation that these levels are well below those produced by FGF doses sufficient to induce mesoderm in an animal cap assay, also indicate that endogenous FGF is not a primary mesoderm inducer. Instead, FGF is a necessary component of mesoderm induction by activin-like growth factors.

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## REFERENCES

**Alessi, D., Smythe, C. and Keyse, S. (**1993). The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and supresses its activation by oncogenic ras in Xenopus oocyte extracts. *Oncogene* **8**, 2015-20.

**Amaya, E., Musci, T. J. and Kirschner, M. W. (**1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-270.

**Biggs, W., Zavitz, K., Dickson, B., van der Straten, A., Brunner, D., Hafen, E. and Zipursky, S. (**1994). The Drosophila rolled locus encodes a MAP kinase required in the sevenless signal transduction pathway. *EMBO J.*

**Blenis, J. (**1993). Signal Transduction via the MAP kinases: Proceed at your own RSK. *Proc. Natl. Acad. Sci. USA* **90**, 5899-5892.

**Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., Zipursky, S. L. and Hafen, E. (**1994). A gain of function mutation in Drosophila MAP kinase activates multiple receptor kinase signaling pathways. *Cell* **76**, 875-888.

**Cai, H., Szeberenyi, J. and Cooper, G. (**1990). Effect of a dominant inhibitory Ha-ras mutation on mitogenic signal transduction in NIH 3T3 cells. *Molec. Cell. Biol.* **10**, 5314-5323.

**Cascio, S. and Gurdon, J. B. (**1987). The initiation of new gene transcription during *Xenopus* gastrulation requires immediately preceding protein synthesis. *Development* **100**, 297-305.

**Charles, C., Sun, H., Lau, L. and Tonks, N. (**1993). The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* **90**, 5292-6.

**Cornell, R. A. and Kimelman, D. (**1994). Activin-mediated mesoderm induction requires FGF. *Development* **120**, 453-462.

**Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (**1994). Activation of MAP kinase is necessary and sufficient for PC12 Differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841-852.

**Cunliffe, V. and Smith, J. C. (**1992). Ectopic mesoderm formation in Xenopus embryos caused by widespread expression of a Brachyury homologue. *Nature* **358**, 427-430.

**Dale, L., Howes, G.,Price, B. M. J. and Smith, J. C. (**1992). Bone Morphogenetic Protein 4: a ventralizing factor in Xenopus development. *Development* **115**, 573-585.

**Dale, L., Matthews, G. and Colman, A. (**1993). Secretion and mesoderm inducing activity of the TGFß related domain of Xenopus Vg1. *EMBO J.* **12**, 4471-4480.

**Dent, P., Haystead, T. A. J., Haser, W., Vincent, L. A., Roberts, T. M. and Sturgill, T. W. (**1992). v-Raf protein kinase activates mitogen activated protein (MAP) kinase kinase in NIH 3T3 cells and *in vitro*. *Science* **257**, 1404-1407.

**Graves, L. M., Northrup, J. L., Potts, B. C., Krebs, E. G. and Kimelman, D. (**1994). Fibroblast growth factor, but not activin, is a potent activator of mitogen-activated protein kinase in Xenopus explants. *Proc. Nat. Acad. Sci. USA* **91**, 1662-1666.

**Gurdon, J. B., Fairman, S., Mohun, T. J. and Brennan, S. (**1985). Activation of muscle specific actin genes in *Xenopus* development by an induction between animal and vegetal cells of a blastula. *Cell* **41**, 913-922.

**Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E. and Maller, J. L. (**1993). Induction of metaphase arrest in cleaving xenopus embryos by map kinase. *Science* **262**, 1262-1265.

**Hartley, R. S., Lewellyn, A. L. and Maller, J. L. (**1994). Map kinase is activated during mesoderm induction in Xenopus laevis. *Dev. Biol.* **163**, 521-524.

**Hemmati-Brivanlou, A. and Melton, D. A. (**1992). A truncated activin receptor dominantly inhibits mesoderm induction and formation of axial structures in Xenopus embryos. *Nature* **359**, 609-614.

**Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C. J. (**1992). Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71**, 335-342.

**Isaacs, H., Pownall, M. and Slack, J. (**1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

**Isaacs, H., Tannahill, D. and Slack, J. (**1992). Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anterioposterior specification. *Development* **114**, 711-720.

**Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E., Hogan, B. J. M. (**1992). DVR-4 (Bone Morphogenetic Protein-4) as a postero-ventralizing factor in Xenopus mesoderm induction. *Development* **115**, 639-647.

**Kimelman, D., Abraham, J. A., Haaparanta, T., Palishi, T. M. and Kirschner, M. W. (**1988). The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science* **242**, 1053-1056.

**Kintner, C. R. and Brockes, J. P. (**1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. *Nature* **308**, 67-69.

**Koster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H. and Knochel, W. (**1991). Bone Morphogenetic Protein 4 (BMP4), a member of the TGF-β family, in early embryos of *Xenopus laevis*: analysis of mesoderm inducing activity. *Mech. Devel.* **33**, 191-200.

**Krieg, P. A. and Melton, D. A. (**1987). An enhancer responsible for activating transcription at the MBT in *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **84**, 2331-2335.

**Kyriakis, J. M., App, H., Zhang, X.-F., Banerjee, P., Brautigan, D. L., Rapp, U. R. and Avruch, J. (**1992). Raf-1 activates MAP kinase-kinase. *Nature* **358**, 417-421.

**LaBonne, C. and Whitman, M. (**1994). Mesoderm induction by activin requires FGF mediated intracellular signals. *Development* **120**, 463-472.

**MacNicol, A. M., Muslin, A. J. and Williams, L. T. (**1993). Raf-1 kinase is essential for early Xenopus development and mediates the induction of mesoderm by FGF. *Cell* **73**, 571-584.

**Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Woude, G. F. V. and Ahn, N. G. (**1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**, 966-969.

**Newport, J. and Kirschner, M. (**1982). A major developmental transition in early *Xenopus* embryos: 1. Characterization and timing of cellular changes at the midblastula transition. *Cell* **30**, 675-686.

**Nieuwkoop, P. D. and Faber, J. (**1967). *Normal Table of Xenopus laevis (Daudin) (*Second edition ed.). Amsterdam: North Holland Publishing Company.

**Rao, Y. (**1994). Conversion of a mesodermalizing molecule, the Xenopus Brachyury gene, into a neuralizing factor. *Genes Dev.* **11**, 939-947.

**Rosa, F. M. (**1989). *Mix.* 1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965-974.

**Schultemerker, S., Smith, J. C. and Dale, L. (**1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of bvg1 and activin - does activin play a role in mesoderm induction. *EMBO J.* **131**, 3533-3541.

**Slack, J. M. W., Isaacs, H. V. and Darlington, B. G.** (1988). Inductive effects of fibroblast growth factor and lithium ion on *Xenopus* blastula ectoderm. *Development* **103**, 581-590.

**Smith, J. C. (**1987). A mesoderm-inducing factor is produced by a *Xenopus* cell line. *Development* **99**, 3-14.

**Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Hermann, B. G.** (1991). Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

**Sun, H., Charles, C., Lau, L. and Tonks, N. (**1993). MKP-1(3CH134) an immediate early gene product, is a dual specificity phosphatase that dephosporylates MAP Kinase in vivo. *Cell* **75**, 487-493.

**Sun, H., Tonks, N. K. and Bar-Sagi, D. (**1994). Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. *Science* **266**, 285-287.

**Symes, K. and Smith, J. C. (**1987). Gastrulation movements provide an early marker of mesoderm induction in *Xenopus laevis. Development* **101**, 339-349.

**Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J. S. (**1992). Ras is essential for nerve growth factor-and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**, 1031-1040.

**Thomsen, G. and Melton, D. (**1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. *Cell* **74**, 433-441.

**Whitman, M. and Melton, D. (**1992). Involvement of p21 ras in Xenopus mesoderm induction. *Nature* **357**, 252-255.

**Wood, K. W., Sarnecki, C., Roberts, T. and Blenis, J. (**1992). Ras mediates nerve growth factor receptor modulation of three signal transducing proteins: MAP kinase, Raf-1, and RSK. *Cell* **68**, 1041-1050.

**Xu, H. P., White, M., Marcus, S. and Wigler, M. (**1994). Concerted action of RAS and G proteins in the sexual response pathways of Schizoszccharomyces pombe. *Mol. Cell Biol.* **14**, 50-58.

**Zaitsevskaya, T. and Cooper, J. (**1992). Developmentally-regulated expression of mitogen-activated (MAP) kinase in *Xenopus laevis*. *Cell Growth and Diff.* **3**, 773-782.

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