A role for MKP3 in axial patterning of the zebrafish embryo

Michael Tsang1, Shingo Maegawa2, Anne Kiang1, Raymond Habas1, Eric Weinberg2 and Igor B. Dawid1,*

1Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA
2Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA
*Author for correspondence (e-mail: idawid@nih.gov).

Summary
Fibroblast growth factors (FGFs) are secreted molecules that can activate the RAS/mitogen-activated protein kinase (MAPK) pathway to serve crucial functions during embryogenesis. Through an in situ hybridization screen for genes with restricted expression patterns during early zebrafish development, we identified a group of genes that exhibit similar expression patterns to FGF genes. We report the characterization of zebrafish MAP kinase phosphatase 3 (MKP3; DUSP6 – Zebrafish Information Network), a member of the FGF synexpression group, showing that it has a crucial role in the specification of axial polarity in the early zebrafish embryo. MKP3 dephosphorylates the activated form of MAPK, inhibiting the RAS/MAPK arm of the FGF signaling pathway. Gain-and loss-of-function studies reveal that MKP3 is required to limit the extent of FGF/RAS/MAPK signaling in the early embryo, and that disturbing this inhibitory pathway disrupts dorsoventral patterning at the onset of gastrulation. The earliest mkp3 expression is restricted to the future dorsal region of the embryo where it is initiated by a maternal β-catenin signal, but soon after its initiation, mkp3 expression comes under the control of FGF signaling. Thus, mkp3 encodes a feedback attenuator of the FGF pathway, the expression of which is initiated at an early stage so as to ensure correct FGF signaling levels at the time of axial patterning.

Key words: MAPK phosphatase, Dorsoventral polarity, Midblastula transition

Introduction
Fibroblast growth factors are a family of over 23 secreted glycosylated proteins that elicit a variety of important processes throughout development (Maciag and Friesel, 1995; Powers et al., 2000). FGFs initiate signaling cascades through the extracellular interactions of ligands with their cognate receptors and heparin sulfate proteoglycans. Binding of FGFs to the FGF receptor tyrosine kinases (RTK) initiates the formation of receptor dimers resulting in the activation of the RAS/MAPK, phospholipase C gamma (PLCγ) and phosphatidylinositol 3-kinase (PI3K) pathways that ultimately regulate gene transcription (Maciag and Friesel, 1995; Powers et al., 2000).

During embryogenesis, FGFs are crucial in the specification of limb outgrowth, the establishment of the isthmic organizer and the patterning of the hindbrain, and further have a role in cell proliferation and survival (Maves et al., 2002; Reifers et al., 1998; Sun et al., 2002; Trumpf et al., 1999; Walshe et al., 2002). FGF proteins may act in a redundant manner so that the simultaneous inactivation of multiple FGF proteins has revealed overlapping functions for these secreted ligands in several vertebrate species. For example, both FGF3 and FGF8 have been shown to be crucial in the formation of the zebrafish otic placode and ventral thalamus (Leger and Brand, 2002; Maroon et al., 2002; Walshe and Mason, 2003). Furthermore, temporal inactivation of FGFs has revealed specific novel functions for FGF3 during the development of the posterior pharyngeal cartilage of the zebrafish ventral head skeleton (David et al., 2002). As the FGF signaling pathway is important for a multitude of developmental processes, tight regulation of its signal intensity and duration is crucial (Evans and Hemmings, 1998; Niehrs and Meinhardt, 2002). Several modulators of the FGF pathway have been identified, including the cytosolic proteins Sprouty and Spred, and the transmembrane protein SEF, all of which function to ensure that proper signaling levels are achieved during development (Fürthauer et al., 2002; Hacohen et al., 1998; Tsang et al., 2002; Wakioka et al., 2001). Sprouty and Spred are general inhibitors of RTK signaling working at the level of RAS/MAPK signaling (Hanafusa et al., 2002; Wakioka et al., 2001). SEF can inhibit both the RAS/MAPK and PI3K branches of the FGF pathway but does not affect pathways initiated by other ligands (Fürthauer et al., 2002; Kovalenko et al., 2003; Tsang et al., 2002). Recently, mouse, chick and Drosophila MKP3s have been shown to be involved in attenuating RAS/MAPK signaling (Dickinson et al., 2002; Eblaghie et al., 2003; Kawakami et al., 2003; Klock and Herrmann, 2002; Rintelen et al., 2003). The role of MKP3 appears to be limited to FGF/RAS/MAPK signaling in the developing chick embryo as mkp3 expression coincides with regions of FGF signaling activity. MKP3 is thus another negative feedback inhibitor of the FGF pathway in the embryo (Eblaghie et al., 2003; Kawakami et al., 2003). Two distinct domains are characteristic of MKP3 proteins, the N-terminal region, which contains a high-affinity ERK/MAPK binding domain, and the C-terminal domain, which constitutes a dual specificity phosphatase (Stewart et al., 1999; Zhang et al., 2003;
MKP3 negatively regulates the MAPK cascade through dephosphorylation of the di-phosphorylated activated MAPK1 and MAPK2 (p42/p44) proteins. Upon binding of phosphorylated MAPKs to the MAPK-binding domain in MKP3, a conformational activation of the C-terminal phosphatase domain is achieved, leading to the inactivation of MAPKs (Camps et al., 1998; Fjeld et al., 2000; Zhao and Zhang, 2001). Thus, the FGF pathway not only induces the transcriptional activation of feedback inhibitors such as sprouty (spry), sef and mkp3, but through the activation of MAPKs, MKP3 protein is also catalytically stimulated to dampen FGF signaling (Camps et al., 1998; Zhao and Zhang, 2001).

In this study, we report the identification of zebrafish mkp3 from a random in situ screen that showed similar expression to fgf3, fgf8, the Ets transcription factors ert and pea3, and the FGF inhibitors sef and spry (Füthauer et al., 2002; Füthauer et al., 2001; Kudoh et al., 2001; Raible and Brand, 2001; Tsang et al., 2002). We determined that MKP3 is a negative feedback regulator of FGF signaling in the zebrafish embryo as it is in mammalian cells, and we show by loss-of-function and gain-of-function experiments that MKP3 is involved in axial patterning. Furthermore, we show that mkp3 expression is initiated by maternal β-catenin activity prior to any detectable FGF/RAS/MAPK signaling. These findings highlight the importance of tight regulation of FGF signal transduction in the early embryo.

Materials and methods
cDNA cloning

Full-length mkp3 cDNA was isolated from an early somitogenesis library as part of an expression screen. The mkp3 sequence is deposited at GenBank under the Accession Number AY329640. Expression constructs were synthesized by PCR amplification of the full open reading frame and insertion into pCS2+ (Turner and Weintraub, 1994) and pCS2+HA. The mkp3::C292S mutant was generated as described in the QuikChange mutagenesis kit (Stratagene), using the primers 5'-GGGTACCTAGCTAGCAGGCA-TCAGTC-3' (forward) and 5'-GACTGATGCTGCAGGAA-GGGAGTGACACACACG-3' (reverse).

Zebrafish fgf3 and fgf8 used in this study were also cloned from the in situ screen (Kudoh et al., 2001). The DNAkt expression construct was generated by subcloning DNAkt cDNA into pCS2+ (Kohn et al., 1996).

Zebrafish maintenance, RNA injections and in situ hybridization

These procedures were performed as described previously (Tsang et al., 2000) with the following modifications for RNA injections. Wild-type zebrafish embryos were injected with mkp3, HA::mkp3 and mkp3::C292S at the 1- to 4-cell stage with 250 pg and 500 pg RNA. To determine the regulation of mkp3 expression, wild-type zebrafish embryos were injected with RNA encoding fgf8 (5 pg), XFD (250 pg), DNAs (250 pg), MKP3 (500 pg), β-actin/S/A mutant (200 pg) and AN-ctcf (250 pg) and injected with 10 pg or 1 pg fgf3; 10 pg, 1 pg or 0.1 pg fgf8; 50 pg β-catenin; or 50 pg β-galactosidase RNA at the 1-cell stage.

Antisense morpholino oligo injections

Morpholino oligos (MO) were obtained from Genetoools as follows: mkp3MO sequence, 5'-GACGAGGTTGAATCTATACAGACAT-3'; ContMO sequence, 5'-CTCTTTACCTCACTAGATTTTTAAT-3'; and mkp3misMO sequence, 5'-GCCTCGCCTTACATTTGCAAATTAT-3'. mkp3MO and mkp3misMO (5 nl) was injected into 2- to 8-cell stage embryos at concentrations ranging from 2-4 mg ml⁻¹, while ContMO was injected at 4 mg ml⁻¹.

Animal cap assay

Xenopus laevis 2-cell stage embryos were injected with activated RAS RNA at 20 pg and mkp3 or mkp3::C292S at 500 pg into each blastomere, as indicated (Whitman and Melton, 1992). Animal caps were dissected at stage 8.5 and cultured until stage 10.5. RNA was isolated from 24 caps for each experiment, and analyzed by RT-PCR as described previously (Tsang et al., 2002).

RT-PCR

Zebrafish embryos were fixed and total RNA was isolated from the indicated stages. Total RNA (2 µg) was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen) and amplified with the following primers: Histone H4, 5'-CAGGAAACCCGCCT-ATCAGTG-3' (forward) and 5'-TTAGAGGGCGCTTCCGGA-GTACC-3' (reverse), 25 cycles; β-actin, 5'-AGACGCTGCTGACTC-3' (forward) and R5'-AGACGCTGCTGACTC-3' (reverse), 25 cycles; mkp3, 5'-CTTCTCACAGGGGTTTTCCGGA-GTACC-3' (forward) and R5'-CTTCTCACAGGGGTTTTCCGGA-GTACC-3' (reverse), 27 cycles.

Cycles conditions were 94°C for 1 minute; 56°C for 1 minute; 72°C for 1 minute.

Immunohistochemical staining and western blotting

Zebrafish embryos were fixed with 4% paraformaldehyde followed by Methanol storage. Embryos were treated with acetone for 10 minutes at –20°C and re-hydrated with a series of PBS/0.1% Tween:Ethanol at room temperature. Embryos were incubated overnight with 1:10,000 anti di-phospho ERK antibodies (TransGen, Beijing) followed by washes with PBS/0.1% Tween. Mouse anti-phosho-ERK (Sigma) antibodies were used at 1:500 and Rabbit anti-ERK (Promega) at 1:1000 dilutions.

Results

Identification of zebrafish MKP3

In an in situ hybridization screen in zebrafish (Kudoh et al., 2001), we identified a gene with similar expression to fgf3, fgf8 and the FGF inhibitors sef and spry; this gene encodes a Map Kinase Phosphatase (MKP) (Fig. 1), a dual specificity protein phosphatase. The most homologous proteins are the recently identified zebrafish MKP3, Takifugu MKP3 and human MKP3, also known as PYST1 (100%, 85% and 81% identity, respectively) (Eblaghie et al., 2003; Groom et al., 1996; Kawakami et al., 2003; Klock and Herrmann, 2002; Mason et al., 1996; Rintelen et al., 2003). Significant homology also exists with other vertebrates including Xenopus (X17C) and chicken MKP3, and also with the Drosophila homolog (Eblaghie et al., 2003; Kawakami et al., 2003; Mason et al., 1996). Both the MAPK/ERK binding and the phosphatase domains are highly conserved with the other vertebrate homologs (Farooq et al., 2001). The phosphatase domain is located within amino acid residues 288-301 and contains the invariant phosphatase motif VXVHCXXXGSRSXT (Wishart et al., 1995).

mkp3 expression and its regulation by FGF signaling

Expression of mkp3 was not detected in the embryo prior to
zygotic transcription, but appeared very soon after the mid-blastula transition (MBT) at the high stage (3.3 hpf) within a localized region that we identified as the prospective dorsal or pre-organizer region by comparison with the ventral gene vent/vegA (Fig. 2A,B) (Kawahara et al., 2000; Melby et al., 2000). The similarity in expression of mkp3 to expression of FGF genes was first noted somewhat later at the oblong stage when both fgf8 and fgf3 are initially turned on within the future dorsal region of the embryo, and continues within the margin at late blastula (Fig. 2C,D; Fig. 3C,D). Later during somitogenesis stages, expression of mkp3 is detected in the presumptive dorsal forebrain, presumptive mid-hindbrain boundary (MHB), rhombomere 4, the developing somites and throughout the caudal region of the embryo (Fig. 2E,F). At 24hpf, mkp3 expression was located within domains of FGF expression, including the optic stalks, the MHB, pharyngeal arches and tail bud (Fig. 2G,H). Because of this similarity we tested whether mkp3 is regulated by FGF signaling. This is the case, as injection of fgf8 RNA into the one-cell embryo led to an expansion of mkp3 expression (Fig. 2L). Conversely, inhibition of FGF signaling through the expression of a dominant-negative FGF receptor, XFD, resulted in suppression of mkp3 expression (Fig. 2K) (Amaya et al., 1991). The regulation of mkp3 expression by FGF is confirmed by the loss of mkp3 mRNA in the MHB of the zebrafish fgf8 mutant, acerebellar (ace), and in the pax2.1 mutant, no isthmus (noi), in which FGF activity within the MHB is lost (Fig. 2M-P) (Lun and Brand, 1998; Reifers et al., 1998).

Fig. 1. Predicted amino acid alignment of MKP3 from human, mouse, Xenopus, Takifugu, zebrafish, Drosophila and chick. Phosphatase and ERK binding domains are underlined by red and blue, respectively. Residues marked in blue asterisks indicate the absolutely conserved amino acids found in all vertebrate MKPs within the ERK-binding domain.
endogenous mkp3 message. In situ staining with the 3′UTR probe produced a similar expression pattern as the full-length mkp3 cDNA probe (Fig. 2Q). Both DNRas and mkp3 injection resulted in a strong suppression of mkp3 expression at 30% epiboly (Fig. 2R,S). To block PI3K signaling, we injected a dominant-negative version of Akt (DNAkt), a downstream effector of the PI3K pathway (Aoki et al., 1998; Jiang et al., 2000; Kohn et al., 1996). Mkp3 expression was mostly unaffected in DNAkt-injected embryos (Fig. 2T), but embryos displayed gastrulation defects similar to what has been described for inhibiting the PI3K pathway in zebrafish (Montero et al., 2003). The fact that ectopic expression of DNRas and mkp3 can block expression of mkp3 supports the idea that the FGF/RAS/MAPK pathway regulates mkp3 within the margin at the 30% epiboly stage (4.7 hpf).

Maternal β-catenin activity initiates mkp3 expression

The initiation of mkp3 expression was remarkable in that transcripts were detected very soon after MBT at the high stage, prior to any known FGF activity in the embryo (Fig. 2B). We confirmed by RT-PCR that mkp3 is activated at the high stage, whereas no maternal RNA was detected (Fig. 3A). To define the temporal activation of FGF signaling we revisited the issue of the initial expression of fgf3 and fgf8. Both genes exhibited weak expression at the oblong stage (3.7 hpf), while mkp3 was detected at the high stage at 3.3 hpf (compare Fig. 2B with Fig. 3C,D). To define the timing of FGF activity in the early embryo, as opposed to gene expression, we used specific antibodies to detect the presence of phosphorylated MAPK (MAPK-p). Through western blotting and immunohistochemical techniques, we determined the onset of FGF activity in the zebrafish embryo at the oblong stage within the prospective dorsal region (Fig. 3B,E,F). This timing is similar to the observations in the early Xenopus embryo, in which FGF signaling is activated after the MBT and before the onset of gastrulation (Schöhl and Fagotto, 2002). These results suggest that the initiation of mkp3 expression does not depend on FGF activity but rather is elicited by a maternal signal.

To confirm that this is the case, we injected XFD to block FGF signaling, and examined embryos fixed at the high and at later stages for the initiation in mkp3 expression. Embryos injected with XFD and fixed at the high stage exhibited normal mkp3 expression (Fig. 3G,H), supporting the view that initiation of this expression is independent of FGF signaling. By contrast, XFD-injected embryos examined at the sphere or dome stage showed a marked reduction in mkp3 expression (Fig. 2J,K; Fig. 3I,J); thus, the FGF-independent period of mkp3 expression is quite short in the post-MBT zebrafish embryo. As mkp3 is activated in the region of the embryo that will give rise to the organizer, the β-catenin signal, which is known to be essential for organizer formation, is thought to be involved in this event (Kelly et al., 2000; Wylie et al., 1996).

To test this prediction, we used several approaches. First, we activated β-catenin signaling by treating early embryos with lithium chloride (LiCl) (Klein and Melton, 1996). LiCl-treated embryos showed a strong expansion of mkp3 expression at the high stage, in agreement with the prediction that stabilization of β-catenin activates mkp3 (Fig. 3L). Likewise, injection of an activated form of β-catenin (β-catenin S7A; this form cannot be phosphorylated and targeted for degradation) led to ectopic activation of mkp3 (Fig. 3M) (Liu et al., 1999). By contrast, a

Given that FGFs can activate several signaling cascades, including the RAS/MAPK and the PI3K pathways, we wanted to determine which signaling cassette is responsible for the regulation of mkp3 expression. We injected RNA encoding a dominant-negative version of RAS (DNRas) and mkp3, which have been shown to block RAS-mediated signaling, to determine if mkp3 expression is under the regulation of the RAS/MAPK pathway (Camps et al., 2000; Camps et al., 1998; Keyse, 2000; Whitman and Melton, 1992). As we injected zebrafish mkp3 RNA, we used a probe derived from the 3′ untranslated region (3′UTR) of mkp3 mRNA to determine the

![Fig. 2](image) Embryonic expression of mkp3 in zebrafish. (A–P) Lateral views, except in D and Q–T, animal view. (A) mkp3 is not expressed as a maternal transcript, but is initiated at the high stage (B). (C,D) At 30% epiboly, expression marks the margin. (E–H) During somitogenesis stages, mkp3 is restricted to domains where FGF genes are expressed, such as anterior dorsal, the MHB, rhombomere 4 and the caudal region. (I) Comparison of mkp3 (orange) with the ventral marker, vega2/vent (blue). (J–P) mkp3 expression is regulated by FGF signaling. (J–L) Sphere stage. Inhibition of FGF activity via ectopic expression of XFD, a dominant-negative FGF receptor, suppresses mkp3 expression (K), while hyperactivation of the FGF pathway through the expression of fgf8 induces an expansion in mkp3 expression (L). (M–P) 24 hpf. Expression of mkp3 is lost in the MHB (arrows) in ace, a fgf8 mutant (N), and in noi, a pax2.1 mutant (P). (Q–T) mkp3 expression is regulated by the RAS/MAPK pathway. The mkp3 3′UTR was used to detect endogenous mkp3 expression at 30% epiboly in uninjected (Q), DNRas- (R), mkp3- (S) and DNAkt (T)-injected embryos. mkp3 expression was suppressed in DNRas and mkp3-injected embryos, but was unaffected in DNAkt-injected embryos.
A role for MKP3 in axial patterning

MKP3, a MAPK phosphatase, plays a role in axial patterning. Dominant-negative form of Tcf3 (DN-tcf3), the transcriptional partner of β-catenin, could suppress the initiation of mkp3 expression (Fig. 3N) (Kim et al., 2000; Molenaar et al., 1996). Finally, we used two mutant zebrafish lines, ichabod (ich) and bozozok (boz), to confirm that mkp3 expression was initiated by the activity of maternal β-catenin (Fekany et al., 1999; Kelly et al., 2000; Koos and Ho, 1999). In the maternal ventralized mutant ich, which exhibits a failure in the nuclear accumulation of β-catenin (Kelly et al., 2000), mkp3 expression was absent within the dorsal region of the embryo at blastula stages (Fig. 3O,P). Activation of mkp3 through β-catenin and FGF are independent events, as injection of fgf8 RNA could induce the expression of mkp3 in the ich mutant embryo (Fig. 3Q).

Fig. 4. Ectopic expression of MKP3 blocks RAS/MAPK signaling. (A) mkp3 expression constructs. Sequence represents the phosphatase active site. (B) Xenopus animal explant assay. Explants injected with activated forms of RAS exhibit induction of Xbra, while co-injection of mkp3 suppresses RAS activity. The inactive mutant construct mkp3::C292S had no effect on the activated form of RAS. (C-J) Ectopic expression of mkp3 or mkp3::C292S can suppress or enhance, respectively, the expression of sef and spry4 at gastrula stage.

Functional role for MKP3 during embryogenesis

To determine the function of MKP3 during development, we engineered expression constructs for the wild type protein and a phosphatase mutant version, MKP3::C292S (Fig. 4A). We assayed the activity of these constructs in Xenopus animal cap explants to determine if MKP3 could block MAPK signaling. Overexpression of wild-type mkp3 could inhibit Xenopus Brachyury (Xbra) mRNA induction by an activated form of RAS (Fig. 4B). The co-expression of mkp3::C292S with
activated RAS did not alter the induction of Xbra, suggesting that this mutant construct was functionally inactive in this assay, similar to a previous report (Mason et al., 1996). We next determined whether ectopic expression of mkp3 could repress the FGF target genes sef and spry4 in the zebrafish embryo. Expression of both sef and spry4 were diminished in mkp3 RNA-injected embryos at the shield stage (Fig. 4D,H), while sef and spry4 were unaffected or in some instances upregulated in mkp3::C292S-injected embryos, suggesting that mkp3::C292S can function in a dominant-negative manner in the zebrafish embryo (Fig. 4F,J). These experiments indicate that ectopic expression of mkp3 can suppress FGF signaling in the early embryo.

It has been documented that ectopic expression of fgf8 in the zebrafish embryo can elicit dorsIALIZED phenotypes, and targeted injections of fgf8 RNA could elicit secondary axis formation, suggesting that activation of the FGF pathway can initiate the formation of an organizer in the zebrafish (Fürthauer et al., 1997). A role for FGF in axis formation is supported by the observation that injection of mkp3 RNA elicited ventralization of the embryo, including a decrease in the size of the eyes from the six-somite stage onwards (compare Fig. 5B with 5A; Table 1). At 24 hpf, mkp3-injected embryos often displayed a loss of anterior structures and in some instances a complete loss of the head, while ventral domains such as the blood islands were expanded as marked by an expansion in the expression of gata1, a blood cell marker (Fig. 5E,H, and not shown; Table 2). Conversely, injection of mkp3::C292S resulted in dorsialized embryos, as seen by an elongation of somite stage embryos (Fig. 5C and Table 1). At 24 hpf, mkp3::C292S-injected embryos showed a marked reduction in gata1-positive cells and a loss of trunk structures, as often noted in dorsIALIZED embryos (Fig. 5F,I; Table 2). These results suggest that the MKP3::C292S protein acts in a dominant-negative manner, as also observed in the expansion of sef and spry4 at the shield stage (Fig. 4F,J). As mkp3 and FGF genes are expressed prior to organizer formation in the early embryo, we observed the expansion of chordin at shield stage (N;O; arrowheads indicate the boundary of chd expression), and expression of neural markers en3 and krox20 (P;Q) at one-somite stage. MHB, mid/hindbrain boundary; r3 and r5, rhombomeres 3 and 5.

Table 1. Phenotypes at 24 hpf of embryos injected with mkp3, HA::mkp3 (ventralised) and mkp3::C292S (dorsalised)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>V4</th>
<th>V3</th>
<th>V2</th>
<th>V1</th>
</tr>
</thead>
<tbody>
<tr>
<td>mkp3 (250 pg)</td>
<td>100</td>
<td>59</td>
<td>9</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>mkp3 (500 pg)</td>
<td>249</td>
<td>111</td>
<td>13</td>
<td>22</td>
<td>53</td>
</tr>
<tr>
<td>HA::mkp3 (500 pg)</td>
<td>116</td>
<td>44</td>
<td>7</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>Mkp3::C292S (500 pg)</td>
<td>248</td>
<td>108</td>
<td>18</td>
<td>40</td>
<td>27</td>
</tr>
</tbody>
</table>

V4 represents the strongest ventralized phenotypes, such that injected embryos do not form any anterior structures, and have expanded somites and blood islands. V3 embryos completely lack forebrain and have expanded blood islands. V2 embryos form small eyes and V1 embryos exhibit mild expansion of blood islands. D4 embryos represent the strongest dorsIALIZED phenotypes in that they lack all trunk domains and exhibit an expanded head region. D3 embryos lack the most posterior tail but contain anterior somites. D2 embryos show a loss of the most posterior tail and D1 represent embryos with smaller ventral tail fins.

Fig. 5. Phenotypes of zebrafish embryos injected with mkp3 and mkp3::C292S RNA. (A–C) Somitogenesis stages of zebrafish embryos un.injected (A), injected with mkp3 (B) or with mkp3::C292S (C). Injected embryos display anterior defects (red arrowhead). (D–I) At 24 hpf, mkp3-injected embryos (E) exhibit anterior truncation and expanded trunk-tail region, while mkp3::C292S-injected embryos show trunk-tail defects (F) (compare with un.injected in D). (G–I) Staining for gata1 at 24 hpf reveals ventralization of mkp3-injected (H) and dorsalization in mkp3::C292S-injected embryos (I); arrows indicate region of gata1 staining (compare with G, un.injected). (J–Q) Early dorsoventral markers are disrupted in injected embryos. bmp4 is activated in mkp3-injected embryos (J,K, 70% epiboly), while chordin expression is suppressed (L,M, shield stage). Conversely, ectopic expression of mkp3::C292S expands expression of chordin at shield stage (N,O; arrowheads indicate the boundary of chd expression), and expression of neural markers en3 and krox20 (P,Q) at one-somite stage. MHB, mid/hindbrain boundary; r3 and r5, rhombomeres 3 and 5.
C292S (500 pg) 32 17 15 (47%) gata1
mkp3 (500 pg) 59 44 15 (25%) bmp4
bmp2b
mkp3 (500 pg) 85 60 15 (29%) mkp3
99 70 25 (30%) chd

phenotype to embryos injected with morpholino (mkp3MO)-injected embryos, exhibiting a similar somite stage, a dorsalized phenotype was observed in mkp3 cognate protein (Nasevicius and Ekker, 2000). At the one-5, as marked by as the putative MHB region and hindbrain rhombomeres 3 and 4, expansion of the ventral markers bmp2b and bmp4 of (compare F with G), and expansion of chordin and fgf8 (arrows) (compare I with H and K with J). Neural markers en3 and krox20 are expanded in mkp3MO-injected embryos (compare L with M).

Table 2. Effects of ectopic expression of mkp3 and mkp3::C292S on dorsoventral markers

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Normal Effect</th>
<th>Comment</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>mkp3 (250 pg)</td>
<td>36</td>
<td>21</td>
<td>15 (42%)</td>
<td>gata1 expanded</td>
</tr>
<tr>
<td>mkp3 (500 pg)</td>
<td>39</td>
<td>4</td>
<td>35 (89%)</td>
<td>gata1 expanded</td>
</tr>
<tr>
<td>mkp3 (500 pg)</td>
<td>99</td>
<td>70</td>
<td>29 (30%)</td>
<td>chd decreased</td>
</tr>
<tr>
<td>mkp3 (500 pg)</td>
<td>85</td>
<td>60</td>
<td>25 (29%)</td>
<td>bmp2b expanded</td>
</tr>
<tr>
<td>mkp3 (500 pg)</td>
<td>59</td>
<td>44</td>
<td>15 (25%)</td>
<td>bmp4 expanded</td>
</tr>
<tr>
<td>C292S (500 pg)</td>
<td>32</td>
<td>17</td>
<td>15 (47%)</td>
<td>gata1 decreased</td>
</tr>
<tr>
<td>C292S (500 pg)</td>
<td>200</td>
<td>103</td>
<td>97 (49%)</td>
<td>krox20 expanded</td>
</tr>
</tbody>
</table>

MKP3 is required for proper axial patterning
We injected antisense morpholino oligonucleotides targeting a region surrounding the start codon of mkp3 to deplete the cognate protein (Nasevicius and Ekker, 2000). At the one-somite stage, a dorsIALIZED phenotype was observed in mkp3 morpholino (mkp3MO)-injected embryos, exhibiting a similar phenotype to embryos injected with fgf8 RNA (Fig. 6B; Table 3) (Fürthauer et al., 1997). The phenotypes observed at 24 hpf in the mkp3MO-injected embryos exhibited a loss of trunk structures, while anterior domains were relatively normal (Fig. 6D,E). To show that the effects of the mkp3MO were specific, we injected embryos with a 5 bp mismatched mkp3 morpholino (MisMO); these embryos were wild type in appearance (Fig. 6A,B; Table 3). Furthermore, we obtained rescue of wild-type appearance by co-injection of mkp3MO and a hemagglutinin-tagged version of mkp3 (HA::mkp3) that is insensitive to mkp3MO (Fig. 6C; Table 3). To determine the early effects of mkp3MO, we analyzed the expression of marker genes at the shield stage. The expression of bmp4 was diminished, whereas the expression of chordin was expanded in these embryos, suggesting that mkp3 is required prior to gastrulation to establish boundaries between organizer and non-organizer domains (Fig. 6F-I; Table 4). At the bud stage, the expression of fgf8 was expanded in the future MHB region in the mkp3MO-injected embryos, and markers such as en3 and krox20 revealed an expansion of neural domains (Fig. 6J-M). Thus, mkp3MO elicited phenotypes similar to those produced by injection of fgf8 and opposite to the effects of mkp3 overexpression. These loss-of-function experiments suggest that MKP3 is required to limit the extent of FGF signaling in the establishment of dorsoventral patterning before and during gastrulation by modulating FGF signaling through the RAS/MAPK pathway.

The role of FGFs in axial patterning
The data presented indicate that β-catenin directly initiates mkp3 expression in the dorsal region of the embryo, and that

Table 3. Injection of mkp3MO results in dorsalisation that can be rescued by HA::mkp3

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Normal</th>
<th>Dorsalised</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>mkp3MO</td>
<td>152</td>
<td>93</td>
<td>39 (28%)</td>
<td>One somite</td>
</tr>
<tr>
<td>misMO</td>
<td>45</td>
<td>43</td>
<td>2 (4%)</td>
<td>One somite</td>
</tr>
<tr>
<td>mkp3MO+HA:mkp3</td>
<td>104</td>
<td>101</td>
<td>3 (3%)</td>
<td>One somite</td>
</tr>
</tbody>
</table>

Fig. 6. MKP3 is required in the early embryo for establishment of axial polarity. (A,D,F,H,J,L) Control MO (contMO) or 5 bp mismatch mkp3 MO (misMO)-injected embryos. (B,C,E,G,I,K,M) mkp3MO-injected embryos. Injection of mkp3MO results in dorsalized embryos (compare B and E with A and D). (C) The mkp3MO effects can be rescued by co-injection of HA::mkp3 that is insensitive to mkp3MO. (F-M) mkp3MO disrupts early dorsoventral patterning of the embryo, as shown by suppression of bmp4 (compare F with G), and expansion of chordin and fgf8 (arrows) (compare I with H and K with J). Neural markers en3 and krox20 are expanded in mkp3MO-injected embryos (compare L with M).
**Table 4. Expression of dorsoventral and neural markers are affected in mkp3MO-injected embryos**

<table>
<thead>
<tr>
<th>mkp3MO</th>
<th>n</th>
<th>50</th>
<th>25</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>ContMO</td>
<td>130</td>
<td>51</td>
<td>79</td>
<td>61</td>
</tr>
<tr>
<td>mkp3MO</td>
<td>172</td>
<td>74</td>
<td>56</td>
<td>32</td>
</tr>
<tr>
<td>misMO</td>
<td>103</td>
<td>103</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mkp3MO</td>
<td>214</td>
<td>105</td>
<td>105</td>
<td>51</td>
</tr>
<tr>
<td>mkp3MO</td>
<td>131</td>
<td>84</td>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>mkp3MO</td>
<td>44</td>
<td>43</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>mkp3MO</td>
<td>158</td>
<td>107</td>
<td>51</td>
<td>32</td>
</tr>
<tr>
<td>misMO</td>
<td>74</td>
<td>74</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 7. Expression of FGF genes in ich mutant embryos and rescue of phenotype by FGF. (A-M) Mutant embryos derived from ich<sup>+/–</sup> females and wild-type embryos derived from ich<sup>+/+</sup> females are shown as animal pole views. Initial expression of fgf3 and fgf8 is strongly reduced at sphere stage in mutant embryos (B,D) compared with wild-type embryos (A,C), where the double arrows mark dorsal expression.**

**(E-M) Expression of fgf3 continues to be reduced in mutant embryos at 30% (E,F) and 50% epiboly (I,J). By contrast, fgf8 expression is detected at 30% epiboly around the margin (G,H), and continues in mutant and wild-type embryos at 50% (K,M). (N-O) Overexpression of fgf3 (compare N with O) or fgf8 (compare N with P) partially rescues ich embryos (asterisks indicates rescued embryos). (Q) Rescue of ich by injection of FGF genes and β-catenin. Both fgf3 and fgf8 injections resulted in partial rescue of mutant embryos. RNA encoding the nuclear localized form of β-galactosidase (nβ-gal) or zebrafish β-catenin was injected into ich embryos as a negative and positive control, respectively. Phenotypic classification was slightly modified from Kelly et al. (Kelly et al., 2000): class 1 embryos are the most ventralized, lacking trunk and head; class 1a develop partial trunk and spinal cord but no hindbrain or more anterior structures; class 2 develop spinal cord and hindbrain but not midbrain, forebrain or eyes; class 3 develop incomplete anterior brain and eye structures; class 4 develop a complete AP axis but no notochord; class 5 appear normal at 24 hpf.**

mkp3 is required to tightly control FGF signaling throughout embryogenesis. To further investigate the role of the FGF pathway in organizer formation and to understand the temporal requirement for FGFs in determining axial polarity, we analyzed the expression of FGFs in ich mutant embryos. As seen for mkp3, initiation of fgf3 and fgf8 was severely affected at the sphere stage in ich mutant embryos (Fig. 7A-D). However, the expression of fgf8 recovers starting at 30% epiboly stage in the margin, while fgf3 expression remains diminished in the mutant embryos (Fig. 7E-M). The expression profile of fgf3 is similar to that described in studies in early Xenopus development where inhibition of maternal β-catenin signaling resulted in a marked reduction of XFGF3 expression (Schohl and Fagotto, 2003). It has been postulated that the marginal activity of nuclear β-catenin is crucial in setting up the program for mesoderm induction through the activation of XFGF3 (Schohl and Fagotto, 2003). Thus, we hypothesized that the dorsal initiation of FGF genes in zebrafish embryos is required for repressing the maternal β-catenin signal in polarity determination, while the later expression of FGF genes in the margin is required for mesoderm induction. To test this hypothesis, we injected fgf3 and fgf8 RNA into ich mutant embryos and scored the ability of these mRNAs to rescue the ventralized phenotype. Although injection of zebrafish β-catenin RNA could fully rescue ich embryos to class 5 (wild type), ectopic expression of fgf3 or fgf8 led to a partial rescue of phenotype (classes 2 and 3) (Fig. 7N-Q). fgf3 or fgf8-injected ich embryos were able to form anterior structures such as eyes and forebrain, but failed to develop any notochord, supporting the view that other factors besides FGFs are also required for dorsal patterning. This is further supported in the fact that injection of higher doses of fgf3 or fgf8 (see Materials and methods) into ich embryos resulted only in
Discussion

Identification of zebrafish mkp3 and its role in axial patterning

In this report we have isolated zebrafish mkp3 through a random in situ hybridization screen. MKP3 acts as a potent negative regulator of the p42/p44 class of MAPKs and is involved in the determination of axial patterning of the early zebrafish embryo. Studies on other MAPKs in zebrafish, notably the p38 stress-activated MAPKs, have shown their importance for regulation of synchronous cleavage of the early embryo (Fuji et al., 2000). Thus, different families of MAPKs serve different functions in embryogenesis. The activity of MKP3 is similar to what has been described in other species, including chick and Drosophila where MKP3 is involved in limb development and ommatidial patterning, respectively (Eblaghie et al., 2003; Kawakami et al., 2003; Rintelen et al., 2003). We provide further information on the role of MKP3 that is part of the FGF/RAS/MAPK pathway by showing that it is required in the early zebrafish embryo to maintain dorsoventral polarity. Expression of mkp3 is maintained by active FGF signaling through the RAS/MAPK pathway. This is in agreement with the early expression of chick MKP3 within the neural plate. Kawakami et al. (Kawakami et al., 2003) have shown that chick Mkp3 expression within the limb bud is regulated by active FGF/Pi3K pathway. Therefore, it is likely that the regulation of mkp3 by FGF signaling is under different signaling arms depending on the context. It has been documented that Mkp3 expression can also be induced directly by retinoic acid (RA) signaling in Xenopus (Mason et al., 1996; Moreno and Kintner, 2004). It has even been postulated that this RA regulation is key to controlling FGF signaling during somite development in Xenopus through the activation of Mkp3 expression (Moreno and Kintner, 2004). It is possible that activation of the RAS/MAPK pathway by members of the receptor tyrosine kinase family other than FGFR can also induce mkp3 expression. During early development, it is clear that FGFRs regulate mkp3 expression, but ligands belonging to the platelet derived growth factor (PDGF) or the insulin growth factor (IGF) families might also contribute to the regulation of mkp3 expression, as they too are expressed early and can activate RAS/MAPK signaling in the embryo (Ayaso et al., 2002; Liu et al., 2002; Mercola et al., 1988; Pera et al., 2003; Pera et al., 2001). However, it is unlikely that PDGF or IGF contribute to the regulation of mkp3 expression in the embryo, as both factors are either more widely expressed or too restricted when compared with the expression domains of mkp3 (Ayaso et al., 2002; Liu et al., 2002).

Disruption of the initial RAS/MAPK signal through the ectopic expression of mkp3 results in ventralized embryos, while knock down of MKP3 protein results in the opposite phenotype. The availability of the ich mutant has allowed us to determine that the initial FGF signal is crucial for axial patterning, while the later expression of FGF genes within the margin is responsible for mesoderm induction and maintenance. As FGF proteins can also activate other signaling pathways besides RAS/MAPK, such as the Pi3K and the PLCγ cascades, the question still remains as to whether these pathways are also required for establishing the dorsal polarity of the embryo. Studies in Xenopus have shown that the Pi3K pathway works in parallel with the RAS/MAPK pathway in mesoderm induction (Carballada et al., 2001). This is also most probably the situation in zebrafish, as the ventralized phenotypes derived from ectopic expression of MKP3 were not fully penetrant (see Table 1). Studies looking at the role of Pi3K in zebrafish gastrulation movements have revealed that early ectopic expression of dominant-negative Pi3K resulted in axial patterning defects (Montero et al., 2003). Finally, a PLCγ zebrafish mutant has been isolated and characterized and shown to be defective in the formation of intersegmental blood vessels (Lawson et al., 2003). There was no obvious early dorsoventral patterning phenotype attributed to the PLCγ mutant and this may be explained by the fact that PLCγ is found as a maternal transcript (N. D. Lawson, unpublished). Future studies may reveal whether PLCγ is also crucial in the FGF pathway for axial patterning.

How FGFs can regulate early dorsoventral patterning is not completely understood. One observation is that FGFs can directly suppress the initiation of bmp4 expression in the early embryo (Fürthauer et al., 1997). Similarly, we show that ectopic expression of mkp3 has the opposite effect in that the expression of bmp4 was expanded towards the dorsal region of the embryo. In addition, FGFs can induce the expression of the BMP inhibitor chd (Koshida et al., 2002; Mitchell and Sheets, 2001), suppressing the activity of any BMP protein that is generated. This provides further protection of the dorsal region from the ventralizing effects of secreted BMPs. We show that modulating the activity of MKP3 within the early zebrafish embryo prior to gastrulation alters the expression of chd. Recently, it has shown that FGF acting through the RAS/MAPK pathway can directly inactivate SMAD1 function. As SMAD1 is one of the main molecules responsible for propagating the BMP signal, this explains how FGFs can directly suppress BMP activity (Kretzschmar et al., 1997; Pera et al., 2003; Sater et al., 2003). Thus, we support the view that FGFs have a two-pronged effect in the suppression of the ventralizing BMPs. FGFs appear to act in concert with bos to directly suppress the initiation of Bmp gene expression within the future dorsal region of the embryo. This provides a basis for maintaining a bias for organizer formation, while the second effect of dorsal FGF activity is to activate the expression of chd.

Maternal β-catenin activity and activation of FGF signaling in the early embryo

Observations in Xenopus point to the initiation of XFGf3 expression within the prospective mesodermal region by maternal β-catenin activity, and suggest that this expression is a key requirement in mesoderm induction and maintenance (Schohl and Fagotto, 2003). We find that the expression of fgf3 is lost in the ich mutant, thus suggesting a possible conservation in the initiation of the FGF signaling cascade in the early embryo.
between zebrafish and Xenopus. The difference is that the initial fgf3 expression in the zebrafish is limited to the future dorsal region of the embryo, while later it is localized more widely within the margin. It is this difference in the initial expression that may account for the difference in the activities of early FGF signaling between the two species. In the zebrafish, ectopic expression of FGFS can dorsalize the embryo, while in Xenopus ectopic FGFs expand mesodermal tissue (Kimelman and Kirschner, 1987; Slack et al., 1987; Fürthuer et al., 1997).

Mutagenesis screen in zebrafish so far have only identified ace/fgf8 and ikarus/fgf24 as mutations within the FGF pathway, and these mutations do not have defects in dorsal/ventral patterning (Reifers et al., 1998; Draper et al., 2003; Fischer et al., 2003), suggesting that the FGF ligands act redundantly.

Although MKP3 acts as a feedback modulator of the RAS/MAPK pathway in most cell types and developmental stages, the maternal β-catenin pathway induces the initial mkp3 expression prior to the time when the FGF/MAPK signal arises in the embryo. It is likely that the activity of maternal β-catenin is responsible for the initiation of the entire FGF signaling pathway, including the activation of FGF genes and the negative feedback modulator mkp3. We propose that mkp3 induction is direct, based on the following observations: (1) expression of mkp3 is unchanged in boc mutant embryos; (2) the presence of multiple putative TCF3/LEF1 sites, the obligate DNA binding co-factors for β-catenin, in the 5′ region of the mkp3 gene (M.T., unpublished); and (3) the timing of the initial expression of mkp3, which is ahead of the earliest MAPK activation in the zebrafish embryo. Thus, we surmise that the requirement for tight regulation of FGF signaling makes it necessary to have the feedback regulator MKP3 in place before the initiation of any FGF signal itself arises in development. This need for precise regulation emphasizes the profound effect that FGF signaling has on specification of axial polarity in the early embryo.

We thank E. Laver, W. Wang, J. Noveral and R. Gill for their help with fish care and technical advice. We are grateful to L. Solnica-Krezel and D. Stainier for mutant zebrafish embryos. We thank X. He, A. Chitnis, M. Whitman, E. Amaya, A. Kawahara, R. Roth and D. Turner for DNA plasmids. S.M. and E.W. are supported by NIH grant RO1 HD39272.

References


