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RESEARCH ARTICLE

Conserved Cross-Interactions in *Drosophila* and *Xenopus* Between Ras/MAPK Signaling and the Dual-Specificity Phosphatase MKP3

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The extracellular signal-regulated kinase (ERK) is a key transducer of the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) signaling pathways, and its function is required in multiple processes during animal development. The activity of ERK depends on the phosphorylation state of conserved threonine and tyrosine residues, and this state is regulated by different kinases and phosphatases. A family of phosphatases with specificity toward both threonine and tyrosine residues in ERK (dual-specificity phosphatases) play a conserved role in its dephosphorylation and consequent inactivation. Here, we characterize the function of the dual-specificity phosphatase MKP3 in *Drosophila* EGFR and *Xenopus* FGFR signaling. The function of MKP3 is required during *Drosophila* wing vein formation and *Xenopus* anteroposterior neural patterning. We find that the expression of the *MKP3* gene is localized in places of high EGFR and FGFR signaling. Furthermore, this restricted expression depends on ERK function both in *Drosophila* and *Xenopus*, suggesting that *MKP3* constitutes a conserved negative feedback loop on the activity of the Ras/ERK signaling pathway. *Developmental Dynamics 232:695–708, 2005.* © 2005 Wiley-Liss, Inc.

Key words: cell signaling; MAPK; vein formation; negative feedback; neural development; Drosophila; Xenopus

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INTRODUCTION

Signal transduction pathways are key elements in the regulation of cell behavior and tissue organization during the development of multicellular organisms. The members of each pathway are linked by molecular interactions, and, in general, they control the activity of transcription factors that contribute to the temporal and spatial regulation of gene expression. The Ras/Mitogen activated protein kinase pathway (Ras/MAPK) is conserved from yeasts to humans and participates in a variety of developmental processes, including mating in yeast, vulval development in *Caenorhabditis elegans* and appendage formation in both vertebrates and invertebrates (Herskowitz, 1995; Simcox, 1997; Martin, 1998; Shilo, 2003; Wang et al., 2000; Zecca and Struhl, 2002a). The Ras/MAPK pathway is also implicated in the formation and progression of multitude of human cancers (Edwards, 1999; Farassati et al., 2001; Porter and Vaillancourt, 1998). The core elements of the pathway include the small GTPase Ras, several serine-threonine protein kinases and several nuclear effectors such as the ETS proteins Pointed and Yan (Baonza et al., 2002; Perrimon, 1994; Rebay and Rubin, 1995; Rebay, 2002; Shilo, 2003). Growth factors, hormones, cytokines, and cellular stress, acting through receptor complexes belonging to different superfamilies, activate specific MAPK proteins. MAPK belong to

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three large families exemplified by p38, JNK, and extracellular-regulated kinase (ERK). ERK proteins are activated by receptors with tyrosine kinase activity, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR), and also by receptors coupled to a heterotrimeric guanine nucleotide binding protein (G-proteins; Rebay, 2002). In general, these receptors are activated upon binding of extracellular signals and initiate a phosphorylation cascade culminating in the activation of ERK by phosphorylation of threonine and tyrosine residues in a conserved domain. Activated ERK proteins, in turn, phosphorylate serine and threonine residues in a variety of target proteins, including cytoskeletal components and transcription factors involved in the response to growth factors (Shilo, 2003).

The regulation of the places where the Ras/ERK signaling pathway is active and the control of appropriate levels of signaling, are two key aspects in the implementation of the pathway biological activities (Casci and Freeman, 1999). This outcome is achieved through several mechanisms, including the regulation of both ligands and receptors expression and the activity of antagonists that can turn down the pathway at multiple levels (Perrimon and McMahon, 1999; Rebay, 2002). Of interest, many of these antagonists are expressed in response to signal activation, and constitute negative feedback loops that modulate the levels of signaling and contribute to signal termination (Perrimon and McMahon, 1999; Rebay, 2002). Examples of antagonist are the secreted protein Argos, which plays a determining role in photoreceptor formation in the *Drosophila* eye, and several *Drosophila* and vertebrate proteins such as the transmembrane protein Kek-1 and the cytoplasmic components Sprouty, Spred, and Sef (Golembo et al., 1996; Ghidlione et al., 1999; Vinos and Freeman, 2000; Wakioka et al., 2001; Yusoff et al., 2002; Ghiglione et al., 2003; Kovalenko et al., 2003). These proteins act at different levels of the FGF and EGF signaling pathways, antagonizing the receptor itself (Kek-1, Sef) or the serine-threonine kinase Raf (Sprouty and Spred).

A key point of regulation of Ras/MAPK signaling involves reversible changes in the phosphorylation of ERK. The kinase activity of ERK depends on the phosphorylation of particular threonine and tyrosine residues, and the opposing effects of ERK kinases (MEK) and ERK phosphatases (MKP) determine the cellular levels of activated ERK. The places of ERK activation during development can be visualized in vivo using antibodies against the phosphorylated protein (dp-ERK; Yung et al., 1997; Bier, 1998). The restricted expression of dp-ERK suggests that many mechanisms operate to generate a dynamic ERK activation/deactivation balance in different tissues and developmental stages. The down-regulation and eventual termination of ERK signaling is brought about by dephosphorylation of either the threonine or tyrosine residues within the ERK activation loop motif (Camps et al., 2000). The elimination of phosphate from these residues depends on the activity of specific phosphatases such as tyrosine phosphatases, threonine phosphatases and dual-specificity phosphatases (DSPs; Camps et al., 2000). The proteins of the DSP family have a characteristic structure of two domains, a N-terminal domain involved in ERK interactions and a catalytic C-terminal domain (Camps et al., 2000). The specificity of binding is determined by the N-terminal part of the protein, which shows two domains of homology found in the yeast phosphatase CDC25 (CH2; Muda et al., 1998). Binding of MKP to its specific MAP kinase triggers the activation of the phosphatase catalytic domain (Muda et al., 1998). Of interest, the expression of several DSPs depends in part on MAP kinase activation, and many of them are transcriptionally up-regulated in response to growth factors, both in cell culture experiments (Boschert et al., 1998; Camps et al., 1998, 2000) and in developing embryos (Dickinson et al., 2002; Eblaghie et al., 2003; Kawakami et al., 2003).

In this work, we have studied the role of the DSP member MKP3 during *Drosophila* wing development and in *Xenopus* anteroposterior (A/P) neural patterning. We have characterized the phenotype of both *Drosophila* and *Xenopus MKP3* loss- and gain-of-function genetic conditions, studied their patterns of expression during normal development and analyzed the functional and regulatory relationships between *MKP3* and the Ras/MAPK signaling pathway. Our results indicate that *MKP3* expression is induced in response to EGFR and FGFR receptor activation in *Drosophila* and *Xenopus*, respectively. Upon induction, MKP3 and phosphorylated ERK (dp-ERK) are expressed in the same territories, suggesting a role of MKP3 in sustaining appropriate levels of ERK phosphorylation. These results implicate a conserved negative feedback loop mediated by *MKP3* on Ras/MAPK signaling and suggests a key role of MKP3 in processes where the level of Ras/MAPK signaling is critical for normal developmental, such as in the formation of the *Drosophila* wing veins and during A/P patterning of the *Xenopus* neuroectoderm.

RESULTS

Isolation and Characterization of Drosophila MKP3 Gain of Function Alleles

Three independent insertions of a P-GS element in the 5′ untranslated region (UTR) region of the *MKP3* gene were obtained in a genetic screen designed to identify genes that, when overexpressed, affect the differentiation of veins. All three insertions ($MKP3^{M76}$, $MKP3^{S}$, and $MKP3^{160}$; Fig. 1) cause very similar phenotypes in combination with a variety of Gal4 lines (see Fig. 2). The phenotypes depend on the temporal and spatial expression of the Gal4 drivers used. Thus, in combinations with the $Gal4\text{-}shv^{3kpn}$ driver, which is expressed only in the developing pupal veins L2, L3, L4, and distal L5 (see Experimental Procedures section), the wings are of normal size and these longitudinal veins fail to differentiate (Fig. 2B). When the expression of *MKP3* is driven in the imaginal disc, such as in combinations with *Gal4-sal* (expressed in a central domain in the wing disc), the resulting phenotypes are milder and affect the veins L2 and L4 (Fig. 2C). Increased levels of *MKP3* in its normal domain of expression (in the combina-

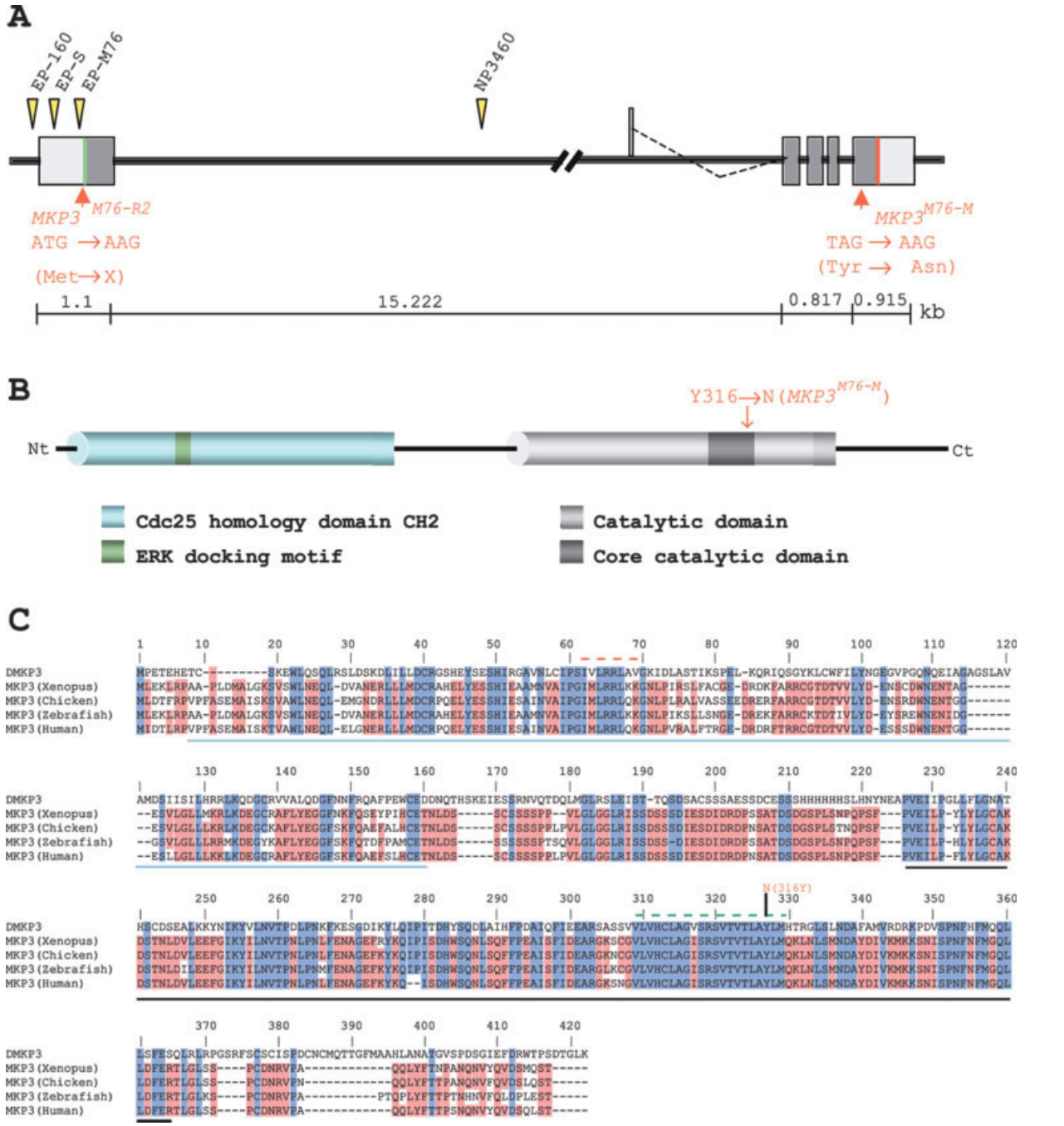

Fig. 1. Molecular map of the *MKP3* genomic region and extracellular signal-regulated kinase phosphatases (MKP) 3 protein domains. **A:** Genomic map indicating the exon–intron structure of the *MKP3* gene and the positions of the P-GS and P-Gal4 insertions, and ethyl methane sulphonate–induced revertants, generated in our analysis. Yellow triangles represent P-element insertion sites. Green and red lines in the mRNA represent the initiation and termination codons, respectively. **B:** Protein domains of MKP3 and position of the amino acid substitution of the $MKP3^{M76\text{-}M}$ allele. The blue region represents the N-terminal region involved in interaction with ERK, and the purple region the catalytic domain of the protein. **C:** Amino acid sequence comparison between *Drosophila* MKP3 with its closest homologues in human, chicken, zebrafish, and *Xenopus*. Light red boxes indicate regions of amino acid identity between at least three of the four vertebrate proteins. Blue boxes represent regions of identity between *Drosophila* MKP3 and at least three vertebrate homologous. The N-terminal and catalytic domains are underlined with blue and black lines, respectively. The position of the amino acid substitution of $MKP3^{M76\text{-}M}$ is also indicated.

tion *Gal4-NP3640/MKP3*^{M76}) also results in a mutant phenotype of loss of veins (Fig. 2D), indicating that both the pattern and level of *MKP3* expression are critical for normal development. When the driver *Gal4-638* (expressed in the wing blade from early stages, not shown) is used, the resulting phenotypes are the complete absence of the wing and its substitution for proximal hinge tissue (Fig. 2E, arrow). Combinations with Gal4 drivers expressed in the

notum region of the wing imaginal disc affect the development of macrochaetae (Fig. 2F) or the formation of the thorax (Fig. 2G). In summary, *MKP3* overexpression in the wing imaginal disc affects developmental processes, such as vein differentiation (see also Rintelen et al., 2003), macrochaetae formation and wing–thorax subdivision, that require EGFR signaling (Diaz-Benjumea and Hafen, 1994; Guichard et al., 1999; Culi et al., 2001; Wang et al., 2000; Cavodeassi et al., 2002; Zecca and Struhl, 2002a,b). Furthermore, the resulting phenotypes of *MKP3* overexpression correspond to strong reductions of EGFR activity, as they are very similar, albeit stronger, than those resulting from ectopic expression of dominant negative forms of the EGFR (data not shown) and loss-of-function alleles of genes of the EGFR partway (Diaz-Benjumea and Hafen, 1994; Guichard et al., 1999; Wang et al., 2000; Culi et al., 2001; Cavodeassi et al., 2002; Zecca and Struhl, 2002a,b). The phenotypic analysis of *MKP3* overexpression suggests that this protein affects EGFR signaling during imaginal development, and indicates that the spatial–temporal pattern and levels of *MKP3* expression are critical for normal EGFR activity.

The Ras/MAPK pathway plays a key role in the formation of veins, and for this reason, the relationships between *MKP3* and other members of the EGFR signaling pathway were further analyzed in the developing pupal veins. We find that the loss of veins characteristic of *MKP3* ectopic expression (Fig. 2B) is retained when *MKP3* is coexpressed with *rhomboid* (Fig. 2H), *EGFR* (not shown), activated-Ras (Fig. 2I), and activated-Raf (not shown). Furthermore, the ectopic and thicker veins differentiating when Rho, EGFR, activated-Ras, and activated-Raf are expressed using the same Gal4 driver (Sotillos and de Celis, 2005) are completely suppressed by coexpression of MKP3 (Fig. 2H,I and data not shown). The only member of the EGFR pathway able to suppress the loss of vein caused by ectopic *MKP3* is the mutant form *sevenmaker* of the gene *rolled* (*rol*^{sem}; Fig. 2J). *Rolled* encodes the *Drosophila* ERK protein, and the mutant form Rol^{sem} affects in vitro its binding to MKP3, behaving as a constitutively activated form (Brunner et al., 1994; Kim et al., 2002). The specific suppression of the *MKP3* overexpression phenotype by

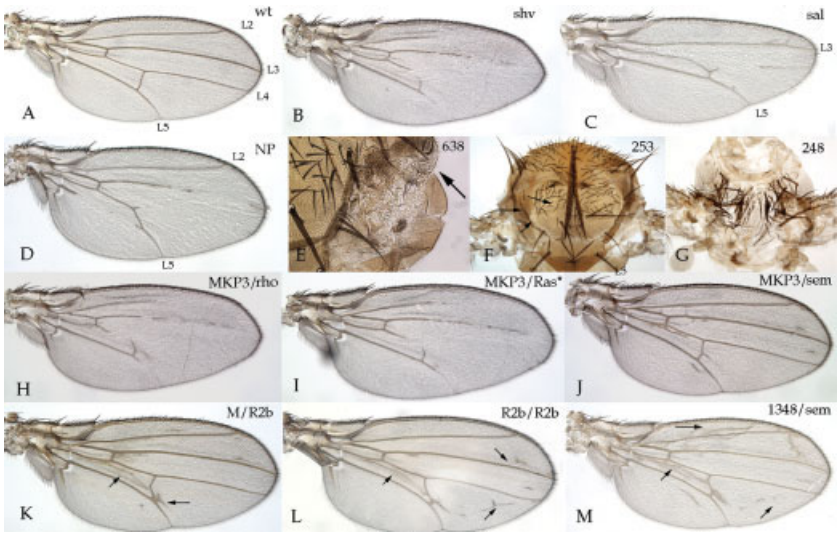

Fig. 2.

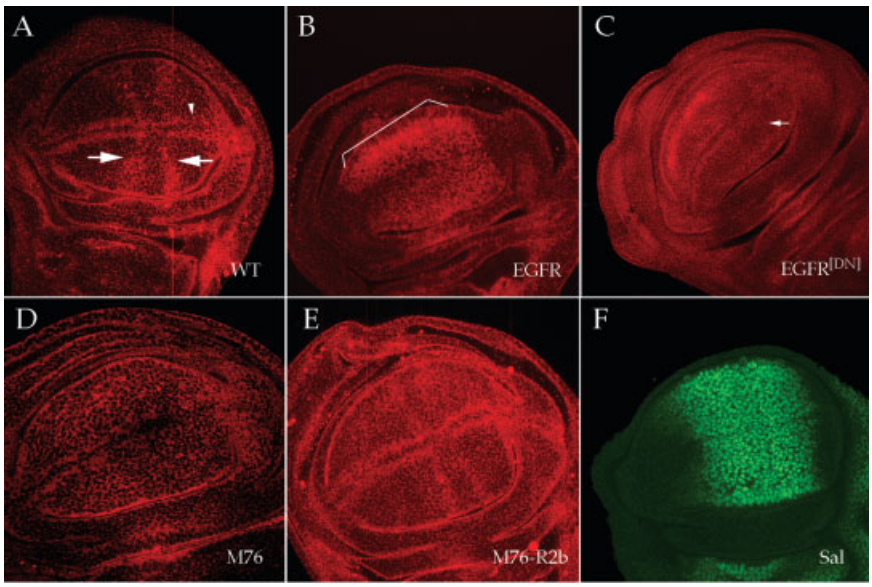

Fig. 3.

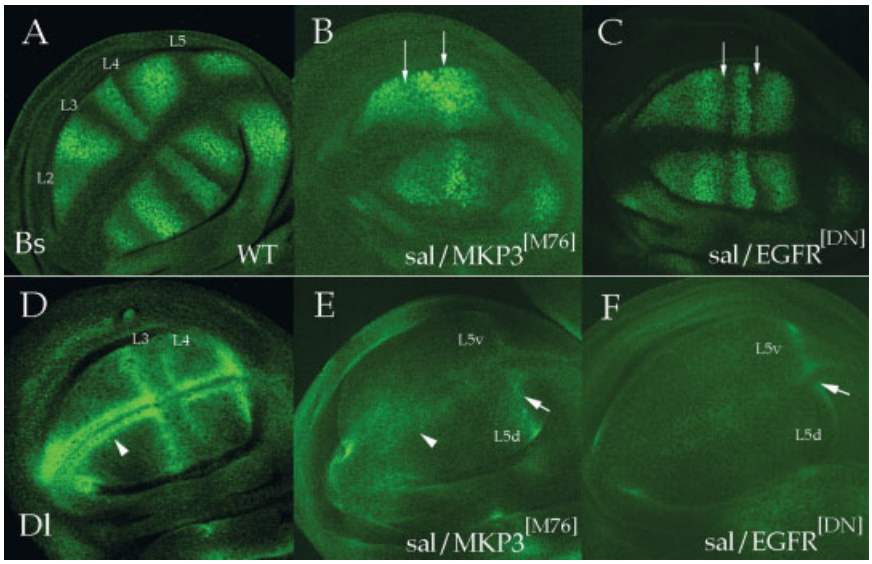

Fig. 4.

rol^{sem} coexpression was also observed by Rintelen et al. (2003) and is compatible with Rolled being the in vivo target of MKP3 activity. Taken together, these results indicate that, during vein differentiation, MKP3 antagonizes the activity of the MAPK protein Rolled.

Isolation and Characterization of *MKP3* Loss-of-Function Alleles

The genetic analysis of gain-of-function conditions in the *MKP3* gene is fully compatible with a role in regulating the activity of ERK by direct dephosphorylation. To identify the requirements of *MKP3* during development, we induced loss-of-function alleles in the gene. Two ethyl methane sulphonate (EMS) -induced revertants ($MKP3^{M76\text{-}M}$ and $MKP3^{M76\text{-}R2b}$; see Experimental Procedures section) of the P-GS insertion $MKP3^{M76}$ cause in the heteroallelic combination $MKP3^{M76\text{-}M}/MKP3^{M76\text{-}R2b}$ the differentiation of ectopic vein stretches in several regions of the wing (Fig. 2K, arrows). The allele $MKP3^{M76\text{-}R2b}$ is homozygous viable with a stronger phenotype of ectopic vein formation (Fig. 2L, arrows). Mitotic recombination clones of the lethal chromosome $MKP3^{M76\text{-}M}$ have normal viability in all wing regions, and only caused the formation of ectopic veins when they appear in the proximal region between the veins L4 and L5 (data not shown). This phenotype is weaker than that of $MKP3^{M76\text{-}R2b}$ homozygous flies, suggesting that the lethality of the $MKP3^{M76\text{-}M}$ chromosome is not related to the *MKP3* gene.

To confirm the presence of point mutations in the $MKP3^{M76\text{-}M}$ and $MKP3^{M76\text{-}R2b}$ chromosomes, we sequenced all the exons and the UTR regions of the *MKP3* transcript. In the $MKP3^{M76\text{-}M}$ mutation, we only identified a T to A transition in position 19018080 (exon 5) that causes the substitution of Tyr316 for Asn (Fig. 1). This Tyr is situated in the catalytic core of the protein, in a region highly conserved in the *MKP3* gene of several species (Fig. 1). The allele $MKP3^{M76\text{-}R2b}$ has only a nucleotide substitution in the first ATG of the coding region, changing the ATG consensus for an AAG codon. The next ATG in frame is located 111 amino acids toward the C-terminal end of the protein, and, if produced, the putative mutant $MKP3^{M76\text{-}R2b}$ protein would lack all of the MKP3 N-terminal domain, which is required for interaction with ERK and phosphatase activation (Fig. 1). Thus, the $MKP3^{M76\text{-}R2b}$ allele most likely corresponds to the null condition of the gene.

The phenotype of homozygous $MKP3^{M76\text{-}R2b}$ flies is very similar to that of genetic combinations in which there is a moderate increase in EGFR signaling, such as in rl^{sem} mutant animals, or when rl^{sem} or *EGFR* are ectopically expressed in the pupal interveins (Fig. 2M, compare with K and L). Taken together, these data suggest that one role of MKP3 is to maintain generalized low levels of EGFR signaling. In addition, the expression pattern of the gene (see below), and the effect of increasing *MKP3* expression in its normal domain, suggest that *MKP3* expression is also critical to maintain appropriate signaling levels

Fig. 2. Phenotypic consequences of changes in *MKP3* activity. **A:** Wild-type (wt) wing showing the longitudinal veins 2 to 5 (L2–L5). **B:** Combination of *Gal4-shv*^{3kpn} (shv) with $MKP3^{M76}$ showing elimination of distal stretches of longitudinal veins L2–L5. The *Gal4-shv*^{3kpn} driver is expressed in the longitudinal veins during pupal development. **C:** Combination of $MKP3^{M76}$ with Gal4-sal (sal), expressed only in the central region of the wing disc, eliminates the veins L2 and L4. **D:** Expression of *MKP3* at higher levels in its normal domain of expression, using a Gal4 line (*Gal4-NP3640*; NP) inserted in the *MKP3* gene, eliminates the veins L3, L4, and L5. **E:** Combination of $MKP3^{M76}$ with *Gal4-638*, which is expressed in the developing wing blade from early stages, causes the substitution of the wing by proximal hinge tissue (arrow). **F,G:** Combination of $MKP3^{M76}$ with the Gal4 lines *Gal4-253* (F; 253) and *Gal4-248* (G; 248) affect the development of macrochaetae (arrows in F correspond to the positions of the missing ASA, PSA, APA, and ADC macrochaetae) or the formation of the thorax (G). The expression of *Gal4-253* is restricted to the proneural clusters. In all cases (B–G), the resulting phenotypes are very similar to the overexpression of a dominant negative version of the EGFR (data not shown) and, therefore, correspond to reductions of EGFR activity. **H–J:** Wing phenotypes of genetic combinations between *Gal4-shv*^{3kpn} $MKP3^{M76}$ and the UAS lines *rho* (*Gal4-shv*^{3kpn} $MKP3^{M76}$/*UAS-rho*; H), *activated Ras* (*Gal4-shv*^{3kpn} $MKP3^{M76}$/*UAS-ras*^{act}; I), and rol^{sem} (*Gal4-shv*^{3kpn} $MKP3^{M76}$/*UAS-rol*^{sem}; J). The thicker veins differentiating when *rho* and Ras^{act} are ectopically expressed (not shown) are suppressed by the coexpression of *MKP3*. Only *UAS-rol*^{sem} is able to suppress the loss of veins caused by *MKP3* misexpression. **K:** Heteroallelic combinations between the *MKP3* loss-of-function alleles $MKP3^{M}$ and $MKP3^{R2b}$ (M/R2b). **L:** Homozygous $MKP3^{R2b}$ wings ($MKP3^{R2b}/MKP3^{R2b}$; R2b/R2b). Ectopic vein tissue differentiates in the proximal region between veins L4 and L5, as well as in other wing territories (arrows in K and L). **M:** Differentiation of ectopic vein tissue after ectopic activation of the epidermal growth factor receptor pathway in the pupal interveins by misexpression of rl^{sem} (1348/sem) using *Gal4-1348* as a driver. Note the similarity in the places of ectopic vein differentiation comparing K and L with M. MKP3, extracellular signal-regulated kinase phosphatase 3.

Fig. 3. Distribution of phosphorylated extracellular signal-regulated kinase (ERK) in the wing imaginal disc. Third instar wing imaginal discs were stained with rabbit anti-dp-ERK. **A:** The expression of dp-ERK in wild-type (WT) discs is maximal in the veins L3 and L4 (arrows) and in the marginal vein (arrowhead). **B,C:** In *Gal4-sal/UAS-EGFR* discs (epidermal growth factor receptor [EGFR], B) the expression of dp-ERK is highly increased in the *sal* domain of expression (bracketed), whereas in *Gal4-sal/UAS-EGFR*^{DN} ($EGFR^{DN}$, C) expression of dp-ERK is diminished (arrow). **D,E:** In *Gal4-sal/* $MKP3^{M76}$ only background levels are detected (M76; D), and in $MKP3^{M76\text{-}R2b}$ homozygous the expression levels are increased (M76-R2b; E). **F:** Expression of the *Gal4-sal* driver (sal) in the wing blade of wild-type discs. The discs shown in A to C and in D,E were stained and processed simultaneously.

Fig. 4. Effects of epidermal growth factor receptor (EGFR), signaling and *MKP3* activity on the expression of vein and intervein markers. **A:** Expression of Blistered (Bs, green) in a wild-type (WT) wing disc, showing the characteristic gaps in the position of the presumptive veins L3, L4, and L5. **B,C:** Expression of Blistered (green) in *Gal4-sal/*$MKP3^{M76}$ (B) and *Gal4-sal/UAS-EGFR*^{DN} (C). In both cases, Bs is now detected in the presumptive L3 and L4 veins (arrows). **D–F:** Expression of Delta (Dl; green) in third instar wing discs of genotypes *Gal4-sal* (D), *Gal4-sal/*$MKP3^{M76}$ (E), and *Gal4-sal/UAS-EGFR*^{DN} (F). The expression of Dl characteristic of the veins L3 and L4 is not detected when $EGFR^{DN}$ or MKP3 are ectopically expressed in the *sal* domain. Also, the expression of Dl abutting the dorsoventral boundary (arrowhead in D) is very reduced in these discs. Dl expression is only detected in the presumptive vein L5 (L5dv; arrows in E and F), which lies outside the domain of *Gal4-sal* expression. MKP3, extracellular signal-regulated kinase phosphatase 3.

in places where the EGFR pathway is more active.

MKP3 Affects ERK Phosphorylation During Vein Differentiation

It has been shown that purified *Drosophila* MKP3 produced in *Escherichia coli* has intrinsic phosphatase activity in vitro and that it can inhibit both the phosphorylation of ERK (encoded in *Drosophila* by *rolled*) and its activity in *Schneider* cells (Kim et al., 2002). Furthermore, MKP3 interacts with ERK through its N-terminal domain, and this interaction is needed for its activity (Kim et al., 2002). No effect of MKP3 on other MAPK proteins such as p38 and JNK were observed in cell culture experiments (Kim et al., 2002). To visualize in vivo the level of phosphorylated ERK (dp-ERK) in different experimental situations where EGFR signaling is affected, we expressed the wild-type receptor (UAS-EGFR), a dominant negative version of EGFR (UAS-EGFR^{DN}), and MKP3 (*MKP3^{M76}*) in the central domain of the wing using *Gal4-sal* as a driver (Fig. 3). In wild-type wing discs, the expression of dp-ERK is maximal in the developing longitudinal wing veins L3 and L4 and in the marginal veins, both in the dorsal and ventral compartments (Gabay et al., 1997; Fig. 3A). This pattern indicates where the activity of the EGFR pathway is maximal, and it is very similar to that of *rhomboid* (*rho*) expression (Sturtevant et al., 1993; Gabay et al., 1997). When the level of EGFR is increased in the *spalt* domain of expression (*Gal4-sal/UAS-EGFR* wing discs) the presence of dp-ERK is highly augmented in this territory (Fig. 3B). Conversely, when either EGFR^{DN} or MKP3 are misexpressed in the *sal* domain, we observe a reduction in the level of dp-ERK (*Gal4-sal/UAS-EGFR^{DN}* wing discs; Fig. 3C) or its absence in the presumptive veins L3 and L4 (*Gal4-sal/MKP3^{M76}* wing discs; Fig. 3D). As expected, the expression of dp-ERK in homozygous *MKP3$^{M76\text{-}R2b}$* wing discs is increased (Fig. 3E), indicating that MKP3 plays a significant role in maintaining appropriate levels of dp-ERK and EGFR signaling in vivo.

Role of *MKP3* During Vein Pattern Formation

To further characterize the role of *MKP3* during vein patterning, we studied the expression of *blistered* (*bs*) and *Delta* (*Dl*), which are known to be regulated by EGFR signaling during imaginal development (de Celis et al., 1997; Huppert et al., 1997; Roch et al., 1998). In these experiments, we compare the effects of ectopic expression of *MKP3* with those of misexpression of EGFR^{DN} (Fig. 4). In wild-type discs, Bs protein expression is restricted to the developing interveins by EGFR-mediated repression (Fig. 4A), and the expression of Dl is maximally localized in the veins L3, L4, and L5 and in the marginal veins due in part to EGFR-mediated activation (Fig. 4D; Kooh et al., 1993; Sturtevant et al., 1993; Fristrom et al., 1994; Montagne et al., 1996; Huppert et al., 1997; Roch et al., 1998). The characteristic down-regulation of Bs in the developing veins L3 and L4 is lost when MKP3 in expressed using Gal4-sal, and only the L5 gap in Bs localization was observed (Fig. 4B). The L5 vein lies outside the domain of Gal4-sal expression (see Fig. 3F). Ectopic expression of EGFR^{DN} has similar consequences, although the effects on Bs protein localization are weaker (Fig. 4C). Ectopic expression of *MKP3* and dominant-negative EGFR cause a similar reduction of Dl expression in the veins L3 and L4 (Fig. 4E,F).

Regulation of *MKP3* Expression by EGFR Activity

An important characteristic of the EGFR pathway is that the levels and pattern of signaling are highly regulated during development (Bier, 1998). Correct modulation of the place and intensity of signaling relies on the restricted expression of several elements of the pathway, such as the ligands Vein and Spitz, as well as of several components involved in the proteolytic processing and activation of the ligands (Rho and Star) (Rutledge et al., 1992; Sturtevant et al., 1993; Simcox et al., 1996; Golembo et al., 1999; Guichard et al., 1999). To study whether *MKP3* expression is related to the activity of the pathway, we analyzed its expression pattern by in situ hybridization in different experimental situations. The *MKP3* transcript is preferentially detected in wild-type wing imaginal discs in two stripes of cells interrupted at the dorsoventral boundary (Fig. 5A). These regions correspond to the developing veins L3 and L4, as revealed by the expression of the MKP3 Gal4 line *NP3640* (Fig. 5B,C). In pupal wings (24 hr after puparium formation [APF]), the maximal levels of expression are present in all longitudinal veins, with low levels detected in most intervein cells (Fig. 5K,L). The specificity of our *mRNA* probe was confirmed in overexpression experiments using the Gal4 lines *sal* (Fig. 5D), *nub* (Fig. 5G), and *shv^{3kpn}* (Fig. 5J) in combination with the P-GS line *MKP3^{M76}*. We also studied the expression of *MKP3* in experimental situations where the activity of the EGFR signaling pathway was altered in the wing blade. In the combinations *Gal4-sal/UAS-EGFR^{DN}* and *Gal4-sal/UAS-EGFR*, we obtained opposite results. Thus, when the activity of the pathway is lowered (*Gal4-sal/UAS-EGFR^{DN}*; Fig. 5E), the levels of *MKP3* expression are very reduced, and, conversely, when the level of signaling is augmented (*Gal4-sal/UAS-EGFR*; Fig. 5F), the expression of *MKP3* is increased in the central domain of the wing. Similar changes in *MKP3* expression were observed in the combinations between *Gal4-nub* and the UAS lines *UAS-EGFR^{DN}* and *UAS-EGFR* (Fig. 5H,I). The correlated changes of EGFR signaling and *MKP3* expression indicate that the transcription of this gene in the developing wing blade is positively regulated by the EGFR signaling pathway.

We also studied the expression of *MKP3* during embryonic development, as the domains of MAPK signaling have been defined accurately in different tissues and stages (Bier, 1998; Gabay et al., 1997). *MKP3* mRNA is uniformly distributed in the syncytial blastoderm (stage 4), indicating an important maternal contribution (Fig. 6A). Later on, as cellularization proceeds, there is a drastic change in mRNA distribution. At early stage 5, mRNA accumulates at the embryonic poles and is absent from the central region (Fig. 6B). This pattern evolves very rapidly with the

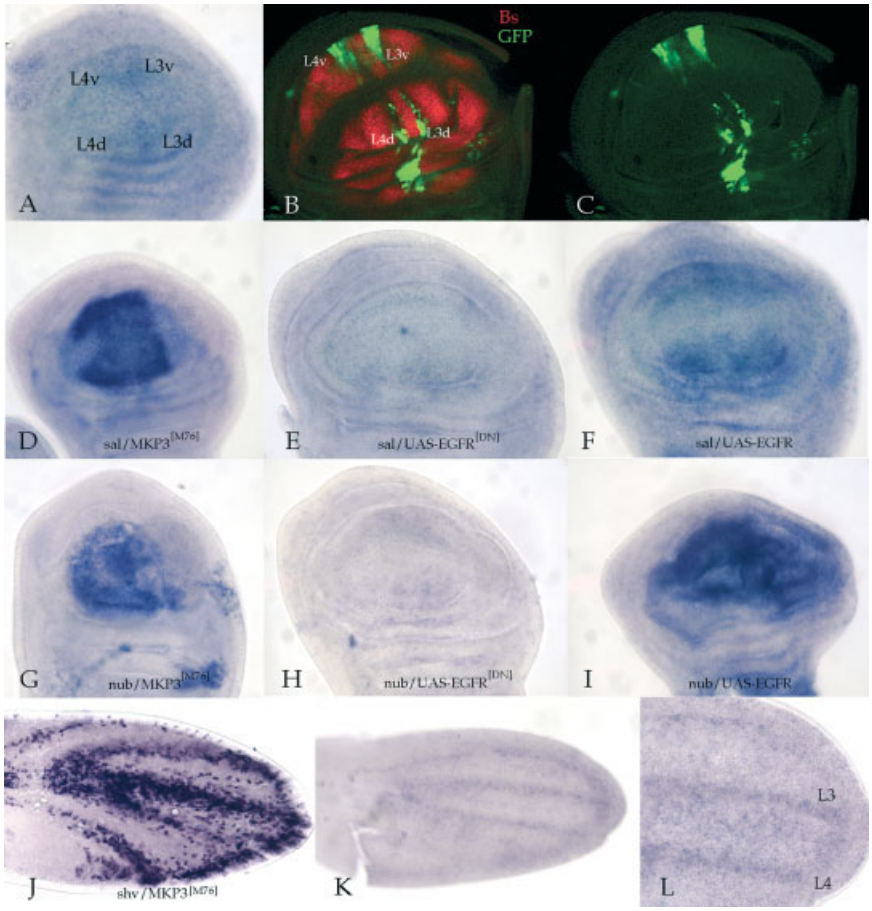

Fig. 5. Expression pattern of *MKP3* in wing discs and pupal wings. **A:** Expression of *MKP3* mRNA in a the wing blade of a third instar wing disc showing preferential accumulation of *MKP3* in the presumptive veins L3 and L4, both in the dorsal (L3d and L4d) and ventral (L3v and L4v) compartments. **B,C:** Expression of the Gal4 line *NP-2436* in the presumptive veins L3 and L4 in flies of genotype *Gal4-NP2436/UAS-GFP*. The expression of Bs in the interveins is in red (B) and the expression of green fluorescent protein (GFP) in the presumptive veins L3 and L4 is in green (B,C). **D–F:** Expression of *MKP3* mRNA in third instar wing discs of genotype *Gal4-sal/MKP3^{M76}* (D), *Gal4-sal/UAS-EGFR^{DN}* (E), and *Gal4-sal/UAS-EGFR* (F). **G–I:** Expression of *MKP3* mRNA in third instar wing discs of genotype *Gal4-nub/MKP3^{M76}* (G), *Gal4-nub/UAS-EGFR^{DN}* (H), and *Gal4-nub/UAS-EGFR* (I). The discs shown in A, D, E, F, G, H, and I were stained with the same probe concentration, the color reaction was stopped at the same time, and the photographs were taken with the same settings. **J:** Expression of *MKP3* mRNA driven by Gal4-shv^{3kpn} in pupal wings 24 hr APF. **K,L:** In wild-type pupae of the same age, the expression of *MKP3* is maximal in the developing pupal veins. L is a higher magnification of K showing the distal ends of veins L3 and L4. EGFR, epidermal growth factor receptor; MKP3, extracellular signal-regulated kinase phosphatase 3.

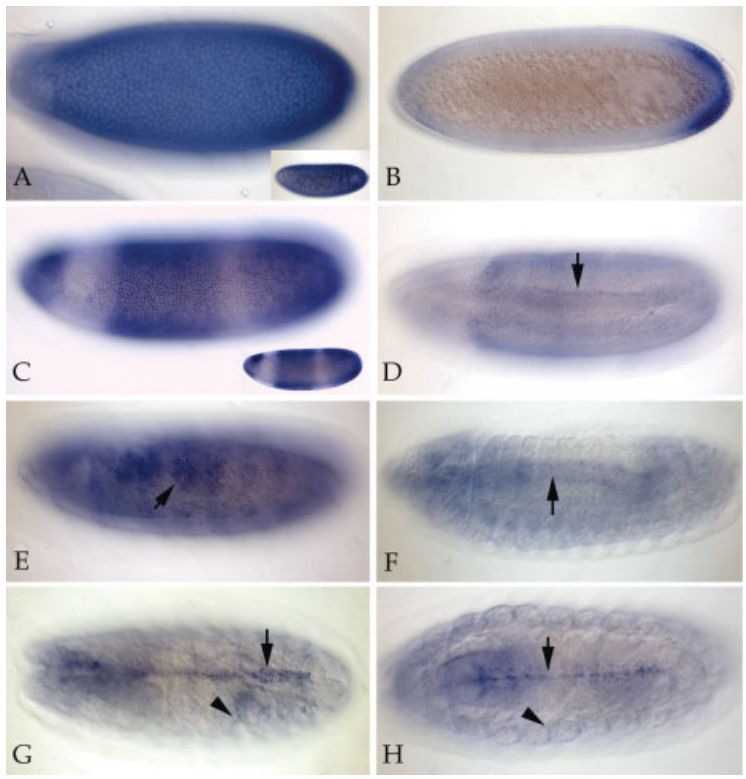

Fig. 6. Expression of extracellular signal-regulated kinase phosphatases 3 (MKP3) during embryogenesis. Embryos at different stages after in situ hybridization with RNA *MKP3* probes. **A:** Lateral view of a syncytial blastoderm showing generalized expression of *MKP3*. The inset shows a deeper focal plane through the embryo. **B,C:** By early stage-5, as cellularization starts, *MKP3* RNA accumulates at the poles of the embryo (B). C: This pattern quickly evolves, as a new domain of expression at the center of the embryo develops. Inset in C shows a deeper focal plane to visualize the ingrowing cell membranes. **D:** Ventral view of a stage 7 embryo showing accumulation of *MKP3* transcripts in the invaginating mesoderm (arrow). **E:** At stage 11, *MKP3* expression is increased at the invaginating tracheal pits (arrow). **F:** Lateral view of a stage 13 embryo. The arrow points to the visceral mesoderm. **G,H:** Dorsal (G) and ventral (H) views of stage 17 embryos showing accumulation of *MKP3* transcripts at the dorsal vessel (arrow in G), in some cells at the midline (arrow in H) and at the apodema (arrowheads in G and H).

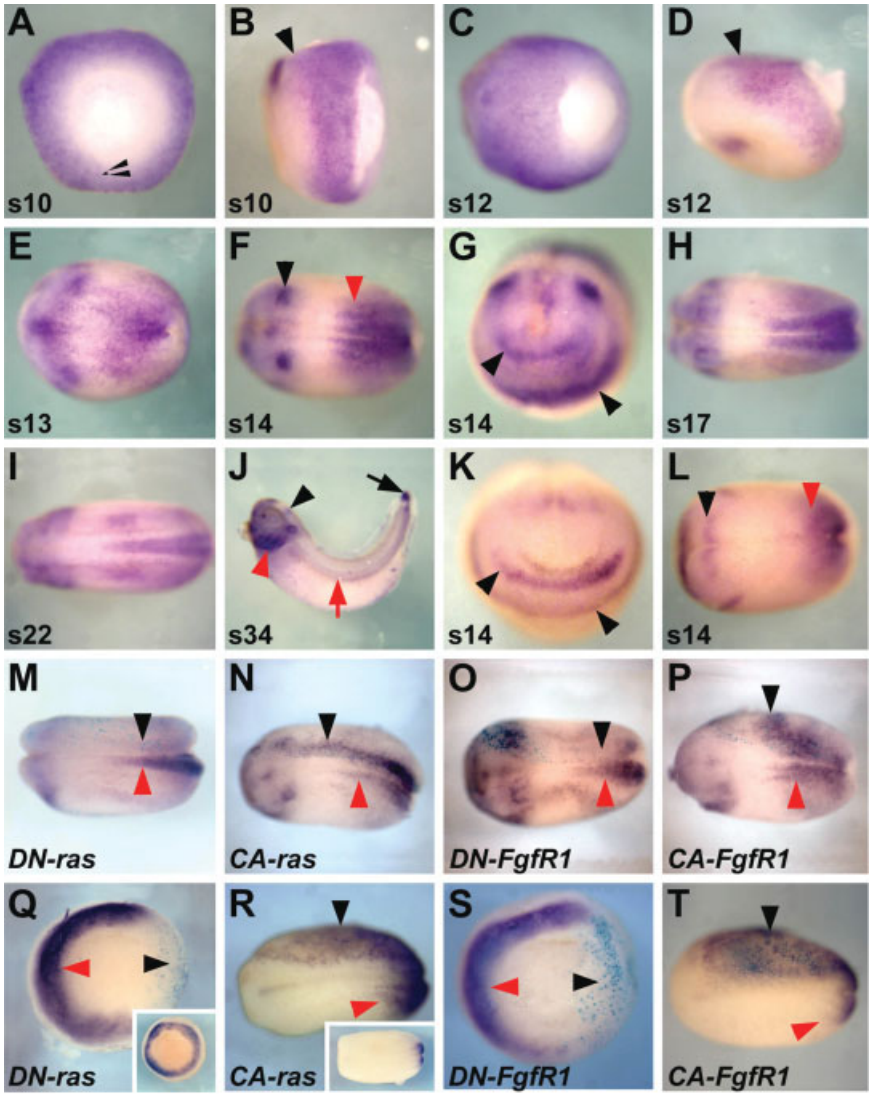

Fig. 7. The fibroblast growth factor mitogen activated protein kinase (FGF/MAPK) pathway regulates *MKP3* expression during *Xenopus* development. All panels show *XMKP3* expression pattern except K,L and Q–T, which show *Fgf8* and *Xbra* expression, respectively. M–T: Injection of different mRNAs along with 300 pg of *LacZ* mRNA in one blastomere at the two-cell stage. The injection side was determined by XGal staining. **A,B:** Vegetal (A) and lateral (B) views of early gastrula embryos (stage 10) show that *XMKP3* is expressed in the mesodermal marginal zone (B) and in the prospective neural ectoderm (B, arrowhead). **C,D:** Vegetal (A) and lateral (B) views of late gastrula embryos (stage 12). *XMKP3* is expressed in all the prospective neuroectoderm (C and arrowhead in D). **E–I:** Dorsal views of different neurula stage embryos show similar *XMKP3* mRNA distribution. At these stages, *XMKP3* expression in the neuroectoderm becomes more restricted, being localized to the posterior neuroectoderm (F, red arrowhead), at the midbrain–hindbrain boundary (F, black arrowhead), and in two horseshoe-shaped bands in the anterior neuroectoderm (G, arrowheads). **J:** Lateral view of tail bud stage 34 embryos. *XMKP3* is strongly detected in the branchial arch region (red arrowhead) and in the tail tip (black arrow). Indeed, these two domains of expression were the only ones detected in a previous report (Mason et al., 1996). In addition, at this stage *MKP3* is also expressed at the midbrain–hindbrain boundary (black arrowhead) and in the somites (red arrow). **K,L:** Anterior (K) and dorsal (L) views of stage 14 embryos showing the expression pattern of *Fgf8*. Note that *Fgf8* and *MKP3* are expressed in similar territories (compare K,G and F,L). **M–P:** Dorsal views of neurula embryos showing *MKP3* expression in embryos injected with different mRNAs. These embryos are at a similar stage to the control embryo shown in H. Red and black arrowheads point at the uninjected (internal control) and the injected sides, respectively. Interfering (M) or increasing (N) Ras activity down-regulates or ectopically activates, respectively, *MKP3* expression. Similarly, interfering (O) or increasing (P) FGF signaling down-regulates or ectopically activates, respectively, *MKP3* expression. The effectiveness of these injections was determined by monitoring *Xbra* expression. **Q–T:** Interfering with FGF/MAPK pathway represses *Xbra* expression at early gastrula (vegetal views, Q,S) while increasing FGF/MAPK activity promotes ectopic *Xbra* expression at neurula stages (dorsal views, R,T). Compare the uninjected (red arrowheads) and the injected (black arrowheads) sides. Insets in Q and R show *Xbra* expression in control embryos at early gastrula and neurula stages, respectively. XMKP3, Xenopus extracellular signal-regulated kinase phosphatase 3.

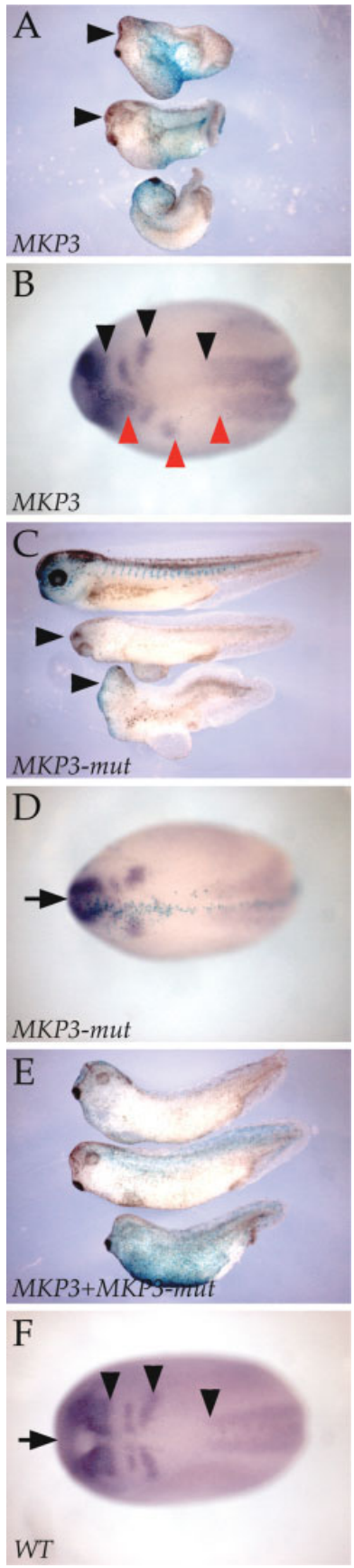

Fig. 8.

formation of a third central domain of expression from 85% to 40% egg length (Fig. 6C). In older embryos, the main places where high levels of *MKP3* transcript accumulate correspond to the invaginating mesoderm (Fig. 6D, arrow), the tracheal pits at stage 11 (Fig. 6E, arrow), the visceral mesoderm at stage 13 (Fig. 6F, arrow), the heart (Fig. 6G, arrow), the midline and the apodema (Fig. 6G,H, arrows and arrowheads, respectively). These territories correspond to places where the activity of several receptor tyrosine kinases (RTK), such as EGFR, Breathless and Torso are required, indicating that there is a good correlation between places of RTK activity and the expression of *MKP3*. Thus, the regulatory relationship between RTK activation and *MKP3* expression observed in the wing disc could also be operative during embryonic development. We have not attempted to analyze the requirements of *MKP3* during embryonic development. To study the functional conservation of *MKP3* regulation and function in other organisms, we characterized the *Xenopus MKP3* homologous gene (*XMKP3*).

Fig. 8. *MKP3* participates in anteroposterior neural patterning. Injection of different mRNAs along with 300 pg of *LacZ* mRNA in one blastomere at the two-cell stage. The injection side was determined by XGal staining. A,C,E: Lateral views of tail bud embryos. B,D,F: Dorsal views of neurula embryos. **A,B:** Overexpression of *MKP3* causes anteriorization of the embryos, as determined by the shortening of the trunk and the slightly enlarged heads (A, arrowheads) and the posterior shift of neural markers (B, compare red with black arrowheads in the injected and control sides, respectively). These markers are *Otx2*, expressed in the anterior-most of the embryos; *krox20*, expressed in rombomeres 3 and 5; and *HoxB9*, expressed in the posterior spinal cord. **C,D:** Overexpression of an mRNA encoding a *MKP3* kinase-dead mutant form (*MKP3-mut*) causes with low efficiency, the impairment of heads structures (C, arrowheads) and reduction of anterior neural markers (D, arrow; compare with an uninjected embryo in F). **E:** Coinjection of *MKP3* and *MKP3-mut* partially rescues the defects observed in *MKP3*-injected embryos. **F:** Wild-type (WT) neurula embryo showing the expression of *Otx2*, *krox20*, and *HoxB9*. MKP, extracellular signal-regulated kinase phosphatases.

Xenopus MKP3 Expression Depends on Ras-Mediated FGF Signaling

The restricted expression of *Drosophila MKP3*, which appears to be controlled by the RAS/MAPK pathway, prompted us to re-examine the expression profile of *Xenopus MKP3*. Previous studies showed that *MKP3* mRNA is localized in restricted domains only at neurula stages (Mason et al., 1996). In contrast, we found that *MKP3* is expressed with a highly restricted pattern all through early development. Thus, at gastrula stages, *MKP3* is found in the mesoderm marginal zone and in the dorsal ectoderm (Fig. 7A–D). During neurulation, *MKP3* mRNA is localized to the posterior neuroectoderm, at the midbrain–hindbrain boundary, and in two horseshoe-shaped domains within the anterior neural plate (Fig. 7E–I). At late neurula and tail bud stages, *MKP3* is also expressed at the branchial arch region, somites, and tail tip (Fig. 7I,J). *MKP3* expression domain is very similar to that of *Fgf8* (Fig. 7K,L), which can signal through the MAPK pathway in vertebrates and invertebrates (Umbhauer et al., 1995; Martin, 1998; Michelson et al., 1998; Borland et al., 2001). Thus, we examined whether the *FGF/MAPK* pathway controls *MKP3* expression. Interfering with MAPK signaling by overexpressing a dominant negative Ras mRNA (*DN*-ras; 1 ng) strongly reduces *MKP3* expression (Fig. 7M, black arrowhead; 58%, n = 41). Conversely, injection of 1 ng of constitutively activated Ras mRNA (*CA-ras*) promotes ectopic *MKP3* expression (Fig. 7N, black arrowhead; 63%, n = 43). We then examined whether modulation of FGF signaling at the receptor level also affects *MKP3* expression. Interfering with FGF signaling by overexpression of a dominant negative FGFR1 mRNA (*DN-FgfR1*; 200 pg) reduces *MKP3* expression in the posterior neuroectoderm (Fig. 7O, black arrowhead; 45%, n = 49). Conversely, overexpression of constitutively activated FGF receptor mRNA (*CA-FgfR1*; 500 pg) caused ectopic expression of *MKP3* (Fig. 7P, black arrowhead; 66%, n = 38). Taken together, our data indicate that FGF, signaling through Ras, regulates the expression of *MKP3*. These results are consistent with recent reports showing that in other vertebrates *MKP3* is expressed in regions in which FGF signaling is active (Klock and Herrmann, 2002; Dickinson et al., 2002; Eblaghie et al., 2003; Kawakami et al., 2003).

MKP3 Negatively Modulates FGF Function in Anterior–Posterior Patterning in *Xenopus*

The expression pattern of *MKP3* and its regulation by the FGF/MAPK pathway suggests that MKP3 is acting as a negative feedback loop of the pathway during *Xenopus* neural development, as it has been reported during mesoderm formation (Umbhauer et al., 1995). During neural development, gain- and lost-of-function analysis of FGF/MAPK indicates its requirement in the generation of posterior neural identities (Mariani and Harland, 1998; Salzberg et al., 1999; Umbhauer et al., 2000; Ribisi et al., 2000). This function involves reprogramming of neural cells with an initial anterior character (reviewed in Stern, 2001). To examine *MKP3* function during neural development, we overexpressed the wild-type form of *MKP3* mRNA (4 ng) (Umbhauer et al., 1995) or the mRNA (4 ng) encoding a phosphatase dead mutant (Mason et al., 1996). This molecule acts as a partial dominant negative form of MKP3 by sequestering MAPK in the cytoplasm (Brunet et al., 1999; Tsang et al., 2004). As previously reported (Umbhauer et al., 1995), most of the embryos injected with *MKP3* mRNA failed to gastrulate due to malformation of mesoderm structures (Fig. 8A, lower embryo; 71%, n = 70) and will not be considered here. Among those embryos that proceeded normally through gastrulation (29%), most of them (12 embryos from 20, 60%) developed shorter A/P axis associated with enlarged anterior structures (Fig. 8A, two upper embryos). Analyses of A/P neural patterning in *MKP3*-injected embryos showed that, in the injected side, the neuroectoderm is anteriorized, as shown by the caudal shift of different neural A/P markers (Fig. 8B, 63% of 19 embryos that proceed normally through gastrulation).

Similar effects have been reported in embryos in which FGF signaling is impaired (Amaya et al., 1991; Holowacz and Sokol, 1999). These results suggest that overexpression of *MKP3* interferes with FGF signaling. The anteriorization observed upon injection of *MKP3* mRNA was not observed upon misexpression of *XMKP3-mut* mRNA. Indeed, most of these injected embryos developed normally (Fig. 8C, upper embryo; 85%, n = 65). However, a small fraction of these embryos showed truncation of anterior structures (Fig. 8C, two lower embryos; 15%). This anterior truncation is also observed at neurula stages by monitoring different A/P neural markers, with anterior markers reduced and posterior genes displaced anteriorly (compare Fig. 8D with a control embryo in Fig. 8F). Similar anterior truncation is observed upon overexpression of several *Fgf* molecules (Lombardo et al., 1998). That the anteriorization observed upon *XMKP3-mut* mRNA injection occurs only in a small fraction of embryos and that even in these embryos not all markers are similarly affected (see in Fig. 8D expression of *HoxB9*, which is not upregulated anteriorly), suggests that XMKP3-mut behave only as a partial dominant negative molecule. This suggestion is consistent with previous data showing that the phosphatase dead mutant form of MKP3 binds to active MAPK, preventing its translocation to the nucleus. This process impairs the activation of nuclear MAPK targets but does not affect that of the cytoplasmic ones (Brunet et al., 1999). Consistent with MKP3-mut behaving, at least in part, as a dominant negative molecule, in embryos coinjected with *MKP3* and *MKP3-mut* mRNAs an increased number of embryos developed normally (Fig. 8E, 43%, n = 60) compared with those injected with *MKP3* alone.

DISCUSSION

The control of appropriate levels of Ras/MAPK signaling is of central importance to determine cell behavior and fate during development. The pathway is regulated at various hierarchical levels, and MAPK proteins are key targets in signal regulation (Garrington and Johnson, 1999; Rebay, 2002). Several mechanisms determine MAPK activity, including scaffolding of MAPK modules in macromolecular signaling complexes, and the balance between the kinases and phosphatases that modify the activity and subcellular localization of MAPK (Garrington and Johnson, 1999; Rebay, 2002). The inactivation of MAPK depends on dephosphorylation of either threonine or tyrosine residues in the MAPK activation loop. The DSP dephosphorylate both serine-threonine and tyrosine leading to reversible inactivation of MAPK. Many aspects of DSP proteins activity and mechanism of action are known by biochemical and cell culture analysis. Thus, individual DSP proteins have specific subcellular localization, they can display high specificity toward particular MAPK targets, and their transcription is generally induced by growth factors, both in cell culture and in vivo (reviewed in Camps et al., 2000). In this work, we have studied the role of the DSP member MKP3, focusing in the biological aspects that determine its function in vivo. To this end, we have used *Drosophila* wing vein formation and *Xenopus* A/P neural patterning as model systems to study the functional requirements of *MKP3* and its relationships with other members of the Ras/MAPK signaling pathway.

MKP3 Antagonizes Specifically Ras/MAPK Signaling

The biochemical analysis of *Drosophila* MKP3 indicated that its activity is specific of the ERK protein Rolled, the MAPK central to both EGF and FGF signaling in *Drosophila* (Kim et al., 2002). This study also showed a cytoplasmic localization of MKP3 in Schneider cells (Kim et al., 2002). The results of our functional analysis of *MKP3* in *Drosophila* are fully compatible with a specific and biologically significant role of the protein in the inactivation of Rolled. Thus, all the phenotypes due to overexpression of *MKP3* are indistinguishable from those caused by inactivation of the Ras/MAPK pathway. These phenotypes are very different to those caused by interference with the Notch, Dpp, Hedgehog, JNK or IP(3)K signaling pathways during imaginal development, indicating high specificity in vivo of MKP3 toward the Ras/MAPK pathway. Furthermore, the effects of *MKP3* overexpression on the transcription of several targets of the Ras/MAPK pathway during vein formation (*bs* and *Dl*) are also similar to those caused by reduction of EGFR activity. The genetic analysis of combinations between *MKP3* and other members of the Ras/MAPK pathway indicates that the target of MKP3 antagonism is, as expected, the MAPK protein Rolled. Therefore, we conclude that MKP3 negatively regulates EGFR signaling by inactivating specifically Rolled during *Drosophila* imaginal development. Rintelen et al. reached independently a similar conclusion in their analysis of *MKP3* in the *Drosophila* eye (Rintelen et al., 2003). During *Xenopus* neural development, we found a similar negative regulation of the FGF pathway by MKP3. Thus, overexpression of *XMKP3* promotes effects consistent with a reduction of FGF signaling such as the reduction of posterior neural structures and the concomitant expansion of anterior ones. Conversely, overexpression of a phosphatase dead mutant form of *XMKP3* cause, to some extend, phenotypes similar to those produced by the ectopic activation of the FGF pathway, namely the suppression of anterior neural structures as a consequence of the expansion of posterior ones.

The cytoplasmic localization of MKP3 in Schneider cells is compatible with a mechanism of MAPK inactivation based on cytoplasmic trapping of this protein. However, a *MKP3* mutant in the catalytic domain ($MKP3^{M76\text{-}M}$) with an intact ERK-interaction domain affects its activity and causes a mutant phenotype, suggesting that direct dephosphorylation is the more likely mechanism of MKP3 antagonism. The second allele we isolated ($MKP3^{M76R2b}$) eliminates the first Met codon and would correspond to the elimination of MKP3 activity. This allele displays a phenotype of ectopic veins indistinguishable from that of other null alleles of the gene (Rintelen et al., 2003). Of interest, the pattern and number of ectopic veins is remarkably similar to that caused by a generalized increase in the activity

of the Ras/MAPK pathway, further indicating the specific requirement of MKP3 in the down-regulation of Ras/MAPK signaling in vivo. The elimination of *MKP3* function is compatible with viability. Furthermore, the phenotype of null alleles is mild when compared with the morphological consequences of other manipulations that activate Ras/MAPK signaling in vivo. Other mechanism involved in signaling down-regulation acting independently of MKP3 might account for the mild phenotypic consequences of *MKP3* elimination. For example, genetic analysis identified the tyrosine-phosphatase PTP-ER as another negative regulator of Rolled (Karim and Rubin, 1999). The phenotype of PTP-ER mutant flies is also very mild, and there is a genetic interaction between this gene and *MKP3*, suggesting some degree of functional redundancy between the two proteins (Karim and Rubin, 1999; Rintelen et al., 2003). Other mechanisms antagonizing ERK activity and Ras signaling might also contribute to regulate negatively the pathway even in the absence of MKP3 and PTP-ER, compensating for the absence of these proteins during wing vein and eye development. In contrast with the moderate effects of *MKP3* elimination, the augmented expression of the gene causes very strong phenotypes typical of Ras/MAPK signaling down-regulation, suggesting that high levels of phosphatase are incompatible with MAPK activity during development.

Ras/MAPK Signaling Regulates *MKP3* Expression

The aim of our analysis was to identify mechanisms regulating MAPK activity during development in vivo. The main aspects of the *MKP3* gene related to its biological activity are its restricted expression and its regulation by Ras/MAPK signaling. Thus, both during embryonic and imaginal development, the expression of *MKP3* is localized to places of Ras/MAPK signaling, such as the terminal poles of the embryo or the developing wing veins. Similarly, the expression of the homologous *Xenopus* gene is also correlated with FGF ligand expression and signaling domains. Moreover, increasing FGF signaling or MAPK activity promotes ectopic *MKP3* expression, while interference with FGF/MAPK pathway suppresses *MKP3* expression. This data indicate that, during *Xenopus* early neural development, *MKP3* is controlled by FGF/MAKP pathway, as has been reported in chick (Eblaghie et al., 2003) and during *Xenopus* mesoderm formation (Umbhauer et al., 1995). The presence of *MKP3* in places of Ras/MAPK signaling is also observed in other vertebrate embryos, indicating a high degree of conservation in the regulation and in vivo function of *MKP3* genes during evolution (Eblaghie et al., 2003). The coincidence between dp-ERK and *MKP3* expression is not always manifested. For example, *MKP3* is not expressed in the apical ectodermal ridge, the tissue where higher levels of dp-ERK are present during vertebrate limb development (Kawakami et al., 2003). Similarly, the expression of *MKP3* in the Drosophila eye disc does not appear to depend on Ras/MAPK activity (Rintelen et al., 2003). These observations indicate that other regulatory inputs besides Ras/MAPK signaling contribute to generate the expression pattern of *MKP3*.

To what extent the regulation of *MKP3* by Ras/MAPK signaling is direct is not known. Recently, it has been proposed that the induction of *MKP3* by ERK is mediated by the PI(3)K pathway in chick embryos and cell culture experiments (Kawakami et al., 2003). Another report, however, suggests that the PI(3)K pathway is not involved in inducing *MKP3* expression in response to FGF (Eblaghie et al., 2003). Our studies in *Xenopus* cannot discriminate whether *MKP3* is induced by the MAPK and/or PI(3)K pathway, because both of them can activate Ras. However, in *Drosophila* the phenotypic consequences of changes in IP(3)K and MKP3 activities are entirely different, because IP(3)K affects only cell growth and size without interfering with pattern formation or cellular differentiation (Stocker and Hafen, 2000). This finding suggests that IP(3)K signaling is not related to the regulation of *MKP3*, at least in *Drosophila* imaginal discs.

In conclusion, the regulation of *MKP3* expression by EGFR and FGFR signaling and the antagonism of MKP3 toward the key pathway transducer ERK, indicates that MKP3 regulates Ras/MAPK signaling acting as a conserved negative feedback loop. In this role, MKP3 plays a comparable function to the specific JNK phosphatase encoded by *puckered* (Martin-Blanco et al., 1988).

EXPERIMENTAL PROCEDURES

Drosophila melanogaster Strains and Phenotypic Analysis

We used the following *Drosophila* UAS lines: *UAS-EGFR*, *UAS-EGFR^{DN}* (Buff et al., 1998), *UAS-ras^{act}* (Brand and Perrimon, 1993), *UAS-sem* (Brunner et al., 1994; Oellers and Hafen, 1996), *UAS-rho* (de Celis et al., 1997), *UAS-Notch^{DN}* (Lawrence et al., 2000), *UAS-tkv^{QD}* (Nellen et al., 1996), *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994), and *UAS-GFP* (Ito et al., 1997). We also used the Gal4 lines *Gal4-shv^{3kpn}* (Sotillos and de Celis, manuscript submitted for publication), *Gal4-nub* and *Gal4-sal* (Calleja et al., 1996), *Gal4-638*, *Gal4-1348* (de Celis, 1997), *Gal4-253* (de Celis et al., 1999), *Gal4-248* (Aldaz et al., 2003), and *Gal4-NP3640* (NP consortium at http://flymap.lab.nig.ac.jp/~dclust/getdb.html). The Gal4-NP3640 line is inserted in the first intron of the *MKP3* gene (NP consortium, see Fig. 1). X-ray induced mitotic recombination clones were generated in flies of genotype *f^{36a}; M(3)i^{55} P[f^{+}]MKP3$^{76\text{-}M}$*. Homozygous *MKP3$^{M76\text{-}M}$* cells were recognized by the presence of the cell marker *forked* (*f*). As a source of P transposase, we used *Δ2-3* and *CyO*, *hop2*. The P-UAS line we used was the double-headed *P-GS* inserted in a *CyO* chromosome (*CyO*, *P-GS*; Toba et al., 1999). Stocks for male recombination were *w; ru e/TM2* and *w; CyO, hop2/If; ru e*. All phenotypes were analyzed at 25°C, and flies were mounted for microscopic examination in lactic acid–ethanol (1:1). Pictures were taken in an Axiophot microscope with a Spot digital camera and processed in Adobe Photoshop.

Generation of *MKP3* Alleles by Male Recombination and by Chemical Mutagenesis

In a mutagenesis screen designed to identify genes affecting vein differentiation (C. Molnar, A. López-Barea and J.F. de Celis, unpublished observations), we isolated three independent P-GS insertions in the 5′ UTR of the *MKP3* gene (see Fig. 1). The three insertions, named $MKP3^{M76}$, $MKP3^{S}$, and $MKP3^{160}$ cause a similar loss-of-vein phenotype in combination with $Gal4\text{-}shv^{3kpn}$ and pupal lethality in combination with *Gal4-638*. Loss-of-function alleles were induced in two different ways: (1) by male recombination (Preston et al., 1996) in male flies of genotype *w; CyO, hop2/+;* $ru^1\ e^{11}$/ $MKP3^{M76}$ crossed to *w*; $ru^1\ e^{11}$ we generated two $ru^1\ MKP3^{M76}$ and five $MKP3^{M76}\ e^{11}$ recombinants. The $ru^1\ MKP3^{M76}$ chromosomes in combination with any Gal4 line are phenotypically normal, whereas all the $MKP3^{M76}\ e^{11}$ recombinants in combination with any Gal4 line retain the $MKP3^{M76}$ gain-of-function phenotype (data not shown). This observation indicates that the gene responsible for the overexpression phenotype lies to the left end (distal) of the P-GS insertion. (2) By chemical mutagenesis, we treated males of genotype w; $MKP3^{M76}/MKP3^{M76}$ with 20 mM EMS according to Lewis and Bacher (1968). Treated males were mass-crossed with *Gal4-638* females and viable progeny of genotype *Gal4-638;* $MKP3^{M76}$/+ were isolated, making the stocks w; $MKP3^{M76\text{-}rev}$/TM2. All flies of *Gal4-638;* $MKP3^{M76}$/+ and *Gal4-638/+;* $MKP3^{M76}$/+ genotype die in the pupal case. Among 3500 progeny (dead pupae that cannot leave the pupal case), we isolated two full revertants named $MKP3^{M76\text{-}M}$ and $MKP3^{M76\text{-}R2b}$. The presence of mutations in both revertants was confirmed by sequencing all exons of the *MKP3* gene (see below).

Molecular Biology

The P-GS lines $MKP3^{M76}$, $MKP3^{S}$, and $MKP3^{160}$ were mapped by inverse PCR following standard procedures. Briefly, genomic DNA from the P-GS strains was digested by using *Hha*I or *Msp*I and the resulting products circularized by using T4-DNA ligase. For the PCR reaction, we used the P-GS specific primers CTTCTTGGCAGATTTCAGTAGTTGC and ATTGCAAGCATACGTTAAGTGGA. For sequencing, we used the primer CGACGGGACCACCTTATGTTA. The resulting sequences were n-Blast against the *Drosophila* genomic sequence at NCBI. The two EMS-induced *MKP3* alleles were sequenced as follows: Genomic DNA was prepared from homozygous flies ($MKP3^{M76\text{-}R2b}$) or homozygous embryos ($MKP3^{M76\text{-}M}$) and used as template for four different PCRs reactions. The primers used were (1) 5′UTR region, CCCGCGCTGCTTGTGTGTGCTCG (upstream) and GGTCTCGTGCTCCGTTTCTGG (downstream); (2) Exon 1, CCAGAAACGGAGCACGAGAC (upstream) and CTTGTAAAGCAACTTACGCGGC (downstream); (3) Exons 2 and 3, GGCTTCAACAATTTCGCCAGGC (upstream) and CTATAAACTGTATGGCATCCGGG (downstream); (4) Exons 4 and 5, GTTTTGAATGTGACACCAGATTTGC (upstream) and CTTCTTCGGCCATCTCCTGATCCCG (downstream). The resulting PCR products were cloned in pGEMTeasy (Invitrogen) and sequenced using T7 and Sp6 vector primers. At least four independent clones resulting from two different PCR reactions made with the same set of primers were sequenced to confirm the presence of nucleotide substitutions. The only differences in sequence we found were T to A transitions in the positions 19035101 (ATG to AAG in $MKP3^{M76\text{-}R2b}$; Met to Lys) and 19018080 (TAC to AAC; Tyr to Asn in $MKP3^{M76\text{-}M}$). Other sequence polymorphism found in our sequences was in position 19018904 in $MKP3^{M76\text{-}R2b}$ (ATC to ATT, both coding Ile).

Drosophila Inmunocytochemistry and In Situ Hybridization

We used rabbit anti-phosphorylated ERK (Cell signaling technologies), mouse monoclonals anti-Dl (Hybridoma bank) and anti-DSRF. Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution). Pupal wings and third instar imaginal discs were dissected, fixed, and stained as described in de Celis (1997). Confocal images were captured using a Bio-Rad confocal microscopy. In situ hybridization in imaginal discs and embryos were carried out as described in de Celis (1997) and Ruiz-Gomez and Ghysen (1993), respectively. Digoxigenin-labeled RNA probes were prepared from the *MKP3* cDNA clone *SD06439* (Research genomics).

Xenopus In Situ Hybridization and X-Gal Staining

The *Xenopus MKP3* probe was prepared by from the Mochii clone XL034f13 linearized with *Eco*RI and transcribed with T7 RNA polymerase using digoxigenin (Roche) as labels. Specimens were prepared, hybridized, and stained as described (Harland, 1991). X-Gal staining was carried out according to Coffman et al. (1993).

In Vitro RNA Synthesis

All DNAs were linearized and transcribed as described (Harland and Weintraub, 1985) with a GTP cap analogue (New England Biolabs). After DNAse treatment, RNA was extracted with phenol-chloroform, column purified, and precipitated with ethanol. mRNAs for injection were resuspended in water. DN-Ras encodes a mutant form of Ras with an Asn at position 17 that generate a dominant negative molecule (Whitman and Melton, 1992). Constitutively activated Ras encodes a mutant form of Ras with Val at position 12 that generate a constitutively active molecule (Whitman and Melton, 1992). Dominant negative FGF receptor type1 also denominated XFD is described in Amaya et al. (1991). Constitutively activated FGF type 1 receptor, also named t-R1, is described in Umbhauer et al. (2000).

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