Mkp3 is a negative feedback modulator of Fgf8 signaling in the mammalian isthmic organizer

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Abstract

The pivotal mechanisms that govern the correct patterning and regionalization of the distinct areas of the mammalian CNS are driven by key molecules that emanate from the so-called secondary organizers at neural plate and tube stages. FGF8 is the candidate morphogenetic molecule to pattern the mesencephalon and rhombencephalon in the isthmic organizer (IsO). Recognizable relevance has been given to the intracellular pathways by which *Fgf8* is regulated and modulated. In chick limb bud development, a dual mitogen-activated protein kinase phosphatase-3 (Mkp3) plays a role as a negative feedback modulator of Fgf8 signaling.

We have investigated the role of Mkp3 and its functional relationship with the Fgf8 signaling pathway in the mouse IsO using gene transfer microelectroporation assays and protein-soaked bead experiments. Here, we demonstrate that MKP3 has a negative feedback action on the MAPK/ERK-mediated FGF8 pathway in the mouse neuroepithelium.
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Introduction

One of the major issues in developmental neurobiology is the study of the molecular and cellular mechanisms that govern, in a very precise manner, the normal regionalization and specification of the vertebrate brain structures. In fact, the specification of brain and body as well as their D/V and A/P axis is dependent upon signals produced at gastrulation and neural plate stages by discrete regions, the primary organizers. These are the anterior visceral endoderm or AVE and the node and its derivatives (reviewed by Beddington and Robertson, 1999; Stern, 2000).

The term secondary organizer is given later, at early neural tube stages, to centers localized along the A/P axis with polarizing and inductive properties. These organizers develop within the broadly regionalized neuroectoderm in genetically defined positions and refine local neural identities (Figdor and Stern, 1993; Joyner et al., 2000; Meinhardt, 1983; Rubenstein et al., 1998; Wassef and Joyner, 1997).

The most studied secondary organizer is the isthmic organizer (IsO) located at the junction between the mesencephalic and rhombencephalic territories in the isthmic constriction or isthmus. The patterning activity of the IsO has been fully demonstrated in many animal models by different experimental, genetic, and molecular approaches

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(reviewed by Echevarria et al., 2003; Martinez, 2001; Nakamura, 2001; Puelles et al., 1996; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Among other signaling molecules expressed in the IsO, FGF8 is so far the only one to have the inductive capability to transform diencephalic tissue into complete ectopic midbrain as well as cerebellar tissue (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). It has been proposed that FGF8 stimulates cell proliferation/differentiation and maintains gene expression pattern essential for IsO activity (reviewed in Echevarria et al., 2003; Liu and Joyner, 2001b; Wurst and Bally-Cuif, 2001). More recently, another role has been attributed to FGF8 signaling in the IsO as a part of a complex gene regulatory network that is essential for neuroepithelial cell survival in rostral rhombencephalon and caudal mesencephalon (Chi et al., 2003). Substantial progress has been made towards understanding the intracellular responses and transductional mechanism of FGF8 signaling pathway. FGF signaling is mediated via tyrosine kinase receptors (FGFRs) that act through a number of transduction pathways, like the highly conserved Ras-ERK mitogen-activated protein kinase (MAPK) (reviewed by Martin, 1998; Niehrs and Meinhardt, 2002) and the phosphatidylinositol 3-kinase (PI3K) signaling cascades. While the activation of the MAPK cascade promotes neural proliferation, differentiation, and apoptosis, activation of the PI3K pathway promotes cell survival (Chen et al., 2000; Ong et al., 2001; Powers et al., 2000). Recent investigations demonstrated that FGF signaling is negatively regulated by complex intracellular systems, which include *Sprouty*, *Sef*, *Spred*, and *FRFS2-alfa* (Furthauer et al., 2002; Hacohen et al., 1998; Kovalenko et al., 2003; Lax et al., 2002; Liu et al., 2003; Tsang et al., 2002; Wakioka et al., 2001). Furthermore, expression of *Sef* and *Sprouty* is induced by activation of the MAP kinases cascade, demonstrating that they operate in negative feedback loops modulating FGF8 intracellular signaling (Furthauer et al., 2002; Minowada et al., 1999; Ozaki et al., 2001; Tsang et al., 2002). More recently, another negative feedback modulator of FGF8 signaling, named MAPK phosphatase-3 (*Mkp3*; Eblaghie et al., 2003; Kawakami et al., 2003), has been described in vertebrate limb bud. *Mkp3* selectively inactivates ERK1/2 class of MAP kinases by dephosphorylation. This leads to catalytic inactivation and thus prevents translocation into the nucleus, ultimately inhibiting ERK1/2-dependent transcription (Camps et al., 1998; Groom et al., 1996; Muda et al., 1996). In mouse embryos, *Mkp3* expression colocalizes with several *Fgf8* expression domains including the IsO (Dickinson et al., 2002; Klock and Herrmann, 2002), which is one of the strongest domains of ERK signaling (Corson et al., 2003).

The study described here was guided by our interest to deepen the knowledge on the nature of these multiple negative modulation mechanisms of FGF8 signaling in the developing mouse brain. In particular, we have examined the role of *Mkp3* in the maintenance and regulation of Fgf8 expression in the IsO and its morphogenetic activity.

Materials and methods

Animal experimentation in this study was carried out in strict accordance with the relevant national and international laws and policies (EEC Council Directive 86/609, OJ L358. 1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals; NIH publication 85-23, 1985; and the Society for Neuroscience, January 1995).

Neural tube dissection and organ explant culture

Timed pregnant ICR mice were sacrificed by cervical dislocation and embryos were dissected from decidual tissue in chilled Hibernate solution (Gibco-Life Technologies). The day of observation of a vaginal plug was defined as E0.5. The developmental stage of the dissected embryos was confirmed by comparing morphological structures with those of the mouse development atlas (Kaufman, 1999). Anterior neural tube of E9.5 embryos was opened along the dorsal midline, placed on polycarbonate membranes, with the ventricular layer facing up, and in vitro cultured in a CO_2 (5%) incubator at 37°C from 7 to 30 h, as described previously (Echevarria et al., 2001).

Implantation of protein-soaked beads

FGF8b-soaked beads were implanted in the neural tube of explant cultures as previously described (Echevarria et al., 2001). Heparin acrylic beads (Sigma) were first rinsed in phosphate-buffered saline (PBS; 0.1 M) four to six times and then soaked in FGF8b solution (1 mg/ml; R&D) for 1 h at 4°C. The beads were then rinsed three times in PBS and thereafter implanted in the neural tube explants. Control beads were soaked only in PBS and implanted in the same manner.

FGF receptor inhibitor (SU5402, Calbiochem) was diluted in DMSO to a final concentration of 4 mg/ml as described elsewhere (Mohammadi et al., 1997; Montero et al., 2001), soaked in ion exchange beads (AG1-X2, Bio-Rad, Hercules, CA) and implanted in neural tube explants. Control beads were soaked in DMSO and implanted in the same manner.

The mitogen-activated protein kinase (MAPK) pathway was inhibited using MEK1 inhibitor (PD98059) and the phosphatidylinositol 3-kinase (PI3K) pathway using the PI3K inhibitor (LY294002) (Cell Signaling Technology, Inc). Both inhibitors were diluted in DMSO to a final concentration of 20 μM and soaked in Affigel blue beads (Bio-Rad), as described for FGF8b-soaked beads. Control beads were soaked in DMSO and implanted in the same manner. The time of incubation for the different beads varied between 7 and 30 h.

Plasmid constructs

A mouse EST (BC003869) containing the entire coding sequence of mouse MKP3 was ordered from RZPD

(www.rzpd.de). The pcDNA6-MKP3/V5-HisC expression vector was constructed by inserting in frame with the V5-His tag from pcDNA6/V5-HisC (Invitrogen), a 0.5-kb *Eco*RI/*Xba*I band, and a 0.7-kb *Xba*I/*Pst*I band from BC003869. The MKP3–siRNA construct was generated by inserting into pSUPER vector, a 19-nt sequence separated by a spacer from the reverse complement of the same sequence (Brummel-kamp et al., 2002). The selection of the 19-nt target sequence was done by scanning the coding sequence of mouse MKP3, 100 nt downstream of the ATG and upstream of the TGA, for 5′AA dinucleotides, and blasting the resulting 21 nt sequences against nr (nonredundant) and mouse EST databases (www.ncbi.nlm.nih.gov/) in order to eliminate sequences with significant homology. The following oligos were used: (siMKP3-fwd: GATCCC**CTTGGAC-GTGTTGGAAGAG**TTCAAGAGA**CTCTTCC-AACACGTCCAAG**TTTTTGGAAA) (siMKP3-rev: AGCTTTCCAAAAA**CTTGGACGTGTTGGAAGAG**-TCTCTTGAA**CTCTTCCAACACGTCCAAG**GGG).

The MKP3–siRNA vector was transformed into SURE competent cells (Stratagene) and the resulting positive clones sequenced to check for the absence of recombination.

Cell culture and siRNA transfection

HeLa cells were cultured in DMEM plus 10% fetal bovine serum (FBS). Cell transfections were done using LF2000 reagent (Invitrogen) and were carried out as described in the manufacturer's protocol. A volume of 1 μl of LF2000 was diluted in 50 μl of DMEM and incubated for less than 5 min at RT before being added to 50 μl of DMEM containing a maximum of 2.0 μg of DNA. This mixture was incubated at room temperature for 20 min and then used to transfect HeLa cells (5×10^4–1×10^5 cells/ml) growing in 12-well plates in a volume of 0.5 ml DMEM medium. These cells were incubated for 2 h, after which the medium was replaced by 1 ml of DMEM with 10% FBS. Cells were then incubated at 37°C, 5% CO_2, and harvested 12, 18, 24, 36, and 48 h after transfection.

Western blot analysis

Protein extracts of sonicated cells were loaded in a 15% polyacrylamide gel and separated by denaturing SDS-PAGE (Laemmli et al., 1970). The separated proteins were then transferred to a nitrocellulose membrane and detected using a mouse monoclonal anti-V5 antibody (Invitrogen) for MKP3-V5.

Micro-electroporation in mouse explant cultures

Electroporation was performed in neural tube explants as shown in Fig. 5E by using a square pulse generator device (UMH University, Spain). DNA [empty pSUPER vector (control) or siRNA (experimental)] was mixed (1:1) with a *Gfp* expression vector (Stuhmer et al., 2002) to a final

concentration of 2 μg/μl in Fast green (Sigma; 1:1000 in distilled water). This DNA was then loaded into glass capillaries (WPI), which were pulled in a horizontal puller (WPI) to a final tip diameter of 2–5 μm. The DNA was injected into the neuroepithelium using a pressure injector (UMH University, Spain) and visualized by fast green. The anode electrode was located inside the glass capillary and the cathode electrode was a rectangular gold plate of 3×4 mm placed under the explant membrane. Electroporation was performed by applying five pulses of 10 ms at a current of 100 μA with intervals of 50 ms, in the isthmic region or ectopically in the rostral mesencephalon (see Figs. 5 and 6). After electroporation, each explant was incubated for 6–24 h. After incubation, the explants were fixed in 4% paraformaldehyde overnight at 4°C before further processing (see below).

In situ hybridization in whole-mount embryos

Whole-mount in situ hybridization (WISH) and antisense probe preparation was carried out as described elsewhere (Belo et al., 1997; Echevarria et al., 2001). In some hybridized embryos, posterior paraffin sections were carried out according to the protocol described elsewhere (Martinez et al., 1999); see Fig. 1). The plasmids containing m*Mkp3*, m*Fgf8*, m*En2*, and m*Gbx2* coding sequences were linearized using *Sal*I, *Not*I, *Cla*I, and *Hin*dIII, respectively, and transcribed using T7 RNA polymerase. For double in situ hybridization, digoxigenin (DIG) and fluorescein (FLUO)-labeled antisense RNA probes were used. The detection of the complementary mRNAs was first done using an anti-DIG alkaline–phosphatase-coupled antibody (Boehringer Manheim), followed by a reaction with the alkaline–phosphatase (AP) substrate nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine salt (BCIP) (Boehringer Manheim) until the appearance of a blue color precipitate. After inactivation of the first antibody, embryos were incubated with the second antibody (antifluorescein alkaline phosphatase-coupled; Boehringer Manheim). This reaction was developed with the red color substrate 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) and BCIP (Boehringer Manheim). Stained embryos were examined and photographed using a Leica MZ12.5 dissection microscope. All the images were processed in Adobe Photoshop 6.0 (Adobe System Inc., Mountain View, CA).

Results

Localization of Fgf8 and Mkp3 in the developing neural tube

We first examined in the mouse neural tube the detailed localization of *Mkp3* expression pattern in comparison to
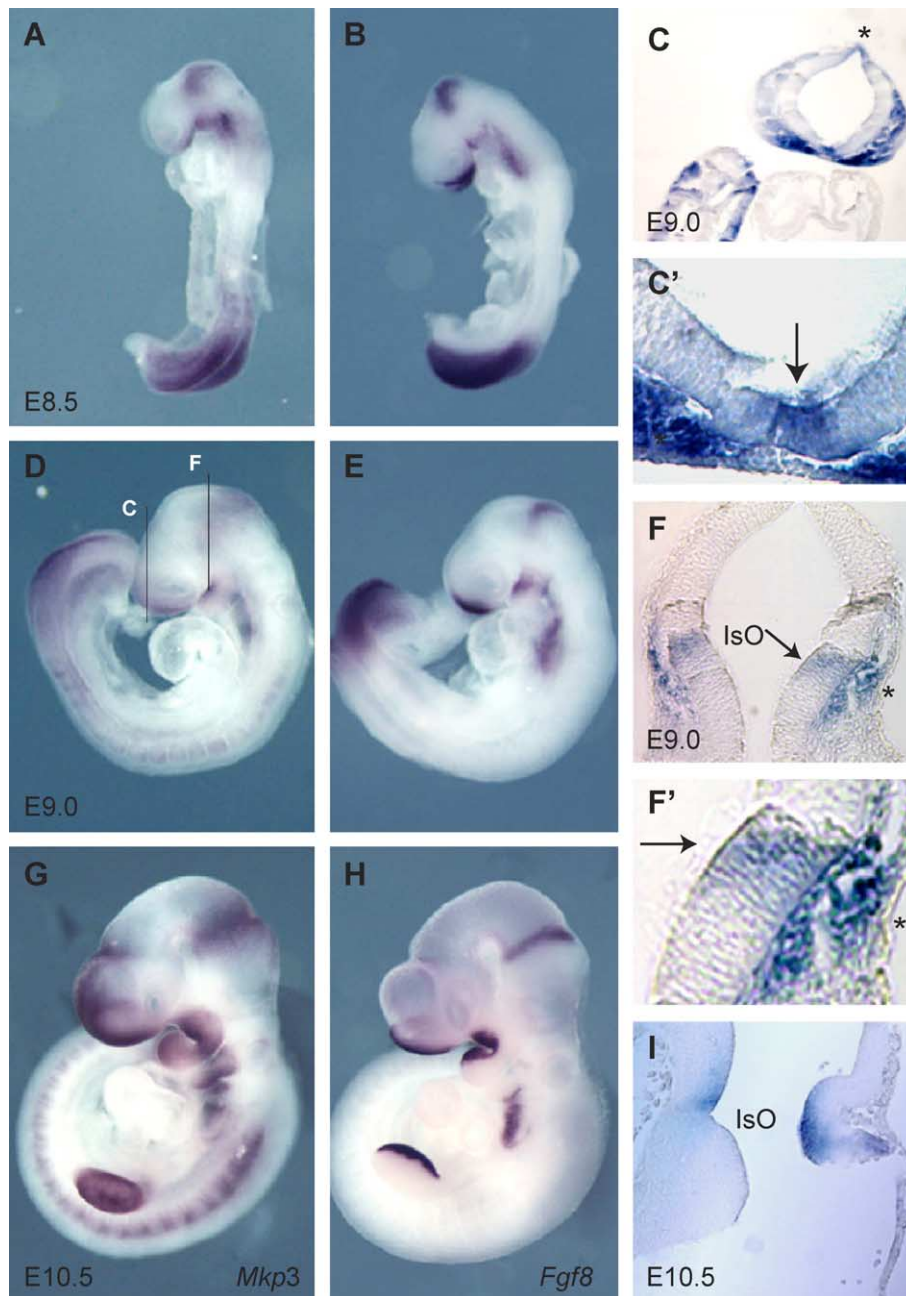
Fig. 1. Comparison of gene expression patterns between *Mkp3* (A, D, and G) and *Fgf8* (B, E, and H) in mouse neural tube during neurulation stages. Note the larger spread and wideness of *Mkp3* expression when compared to the *Fgf8* in the domains of coexpression. Transversal (C, C', F, and F') paraffin sections of an E9.0 mouse embryo after *Mkp3* detection by in situ hybridization (see section levels in D). The black asterisk in C indicates the expression pattern of *Mkp3* in the roof plate (commissural plate). (C') Magnification from the ventral part of C, in which the white asterisk marks the mesenchyme-positive expression domain, and the arrow indicates the floor plate expression. (F) The neuroepithelial (indicated by an arrow) and the mesenchymal (indicated by an asterisk) *Mkp3* expression pattern in the IsO. (F') Magnification of the IsO shown in F. (I) Sagittal section of E10.5 mouse embryo showing the expression of *Mkp3* in the isthmic constriction (paramedian plate) as wells as in the primordium of the cerebellum.

that of *Fgf8* (Fig. 1). In E7.5 mouse embryos *Mkp3* transcripts are already visible in the primitive streak and in the yolk sac (not shown; Dickinson et al., 2002; Klock and Herrmann, 2002). At stage E8.5, *Mkp3* and *Fgf8* expressions colocalized along the neural tube, in particular, in the isthmic region and in the anterior neural ridge (ANR), as well as in other non-neural tissues like the branchial arches and the tail bud (Figs. 1A and B). From E8.5 to

E10.5 (Figs. 1C–F), the expression patterns of *Mkp3* and *Fgf8* were maintained in the same locations although the expression domain of *Fgf8* became more restricted than that of *Mkp3*. In the IsO, *Mkp3* expression pattern extended from the isthmic domain into both the caudal mesencephalon and the rostral rhombencephalon (Figs. 1D, F, F', I, and 2A), while *Fgf8* was only detected as a narrow transversal ring stripe in the isthmus (Figs. 1E and H). At E9.0 in the
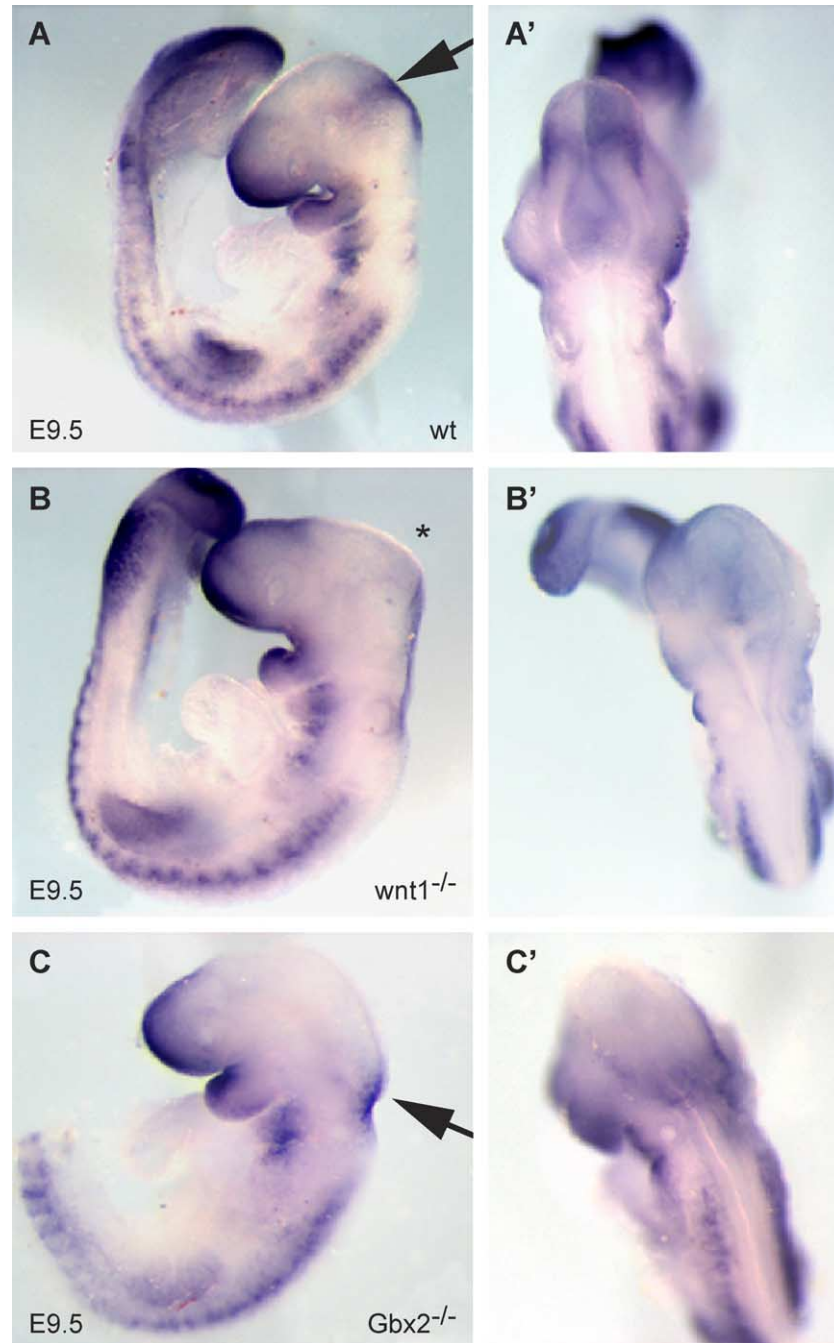
Fig. 2. *Mkp3* expression pattern in modified genetic background contexts. Lateral (A, B, and C) and dorsal (A′, B′, and C′) views of E9.5 mouse embryos after in situ hybridization using an *Mkp3* probe. Comparison of *Mkp3* gene expression in wild type (wt; A and A′), Wnt1$^{-/-}$ (B and B′), and Gbx2$^{-/-}$ (C and C′) embryos. The arrows indicate the position of the isthmic organizer. The asterisk indicates the absence of *Mkp3* in a *Wnt1* knockout mouse embryo. Note the caudalization and reduction of *Mkp3* gene expression in *Gbx2*$^{-/-}$ when compared to the wt situation.

ANR, *Mkp3* transcripts expanded from the ANR towards the dorsal part of the telencephalon (Figs. 1C and D) while *Fgf8* was restricted to the ANR (Fig. 1E). At E9.5 and E10.5, *Mkp3* expression expanded to the rostral roof plate of the diencephalic central limit, the zona limitans intra-thalamica (ZLI; Figs. 1G and 2A), while *Fgf8* expression was only detected along the commissural plate (roof plate of the telencephalon) and in the anterior rostral roof plate of the ZLI (Fig. 1H; see also Fig. 2 in Echevarria et al., 2003).

From E9.5 onwards, *Mkp3* expression was observed in other non-neural tissues such as the limb bud, the branchial arches, the tail bud, and in the mesenchyme surrounding *Mkp3*-positive neuroepithelial cells (Figs. 1D, G, and 2A; see asterisks in Figs. 1C′, F, and F′; Eblaghie et al., 2003; Kawakami et al., 2003). Interestingly, *Mkp3* is expressed in the somites from E8.5 onwards, while *Fgf8* expression was not detected there (Figs. 1 and 2; Dickinson et al., 2002; Klock and Herrmann, 2002). In E9.5 brain explant cultures

as well as in sections of uncultured embryos, an expression pattern not detected by whole-mount in situ hybridization was observed (Figs. 3–5). *Mkp3* was expressed in the floor plate/paramedian plate throughout the neural tube (Fig. 1C, C′, and I). The transcript was localized at the level of rhombomeres 2/3 and continued rostrally through the floor/paramedian plate ending in the anlage at the retromammillar region, just at the rostral edge of the notochord (therefore, along the epichordal plate; see arrow in Fig. 3A). This expression was also detected in the floor plate of the spinal cord, although less intense. Thus, during the early development of the neural tube, both *Mkp3* and *Fgf8* are expressed in the same adjacent domains in the neuroepithelium, particularly in secondary organizers, which suggests a functional related role.

To further address whether *Mkp3* and *Fgf8* are functionally related in the neural tube, we analyzed the expression pattern of these genes in generated mouse mutant lines (Fig. 2). Wnt1 transcription factor is essential for normal midhindbrain development since in $Wnt1^{-/-}$ embryos by E10.0 the IsO is absent (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990). In $Gbx2^{-/-}$ mice, the *Fgf8* expression indicates that the IsO suffers a reduction and caudalization (Millet et al., 1999; Wassarman et al., 1997). By means of whole-mount in situ hybridization (WISH), we observed (as expected) that E9.5 $Wnt1^{-/-}$ mice lack the isthmic expression domain of *Mkp3* that in the wild type is marked by *Fgf8* expression (Fig. 2B); although in the forebrain *Mkp3* expression pattern was maintained. Also, in $Gbx2^{-/-}$ mice, *Mkp3* was found in the IsO caudal to its normal location and reduced in intensity (Figs. 2C and C′), as was the case for the *Fgf8* expression (Wassarman et al., 1997), as expected. Thus, these results indicate a relation between the expression patterns of both *Mkp3* and *Fgf8* in the IsO.
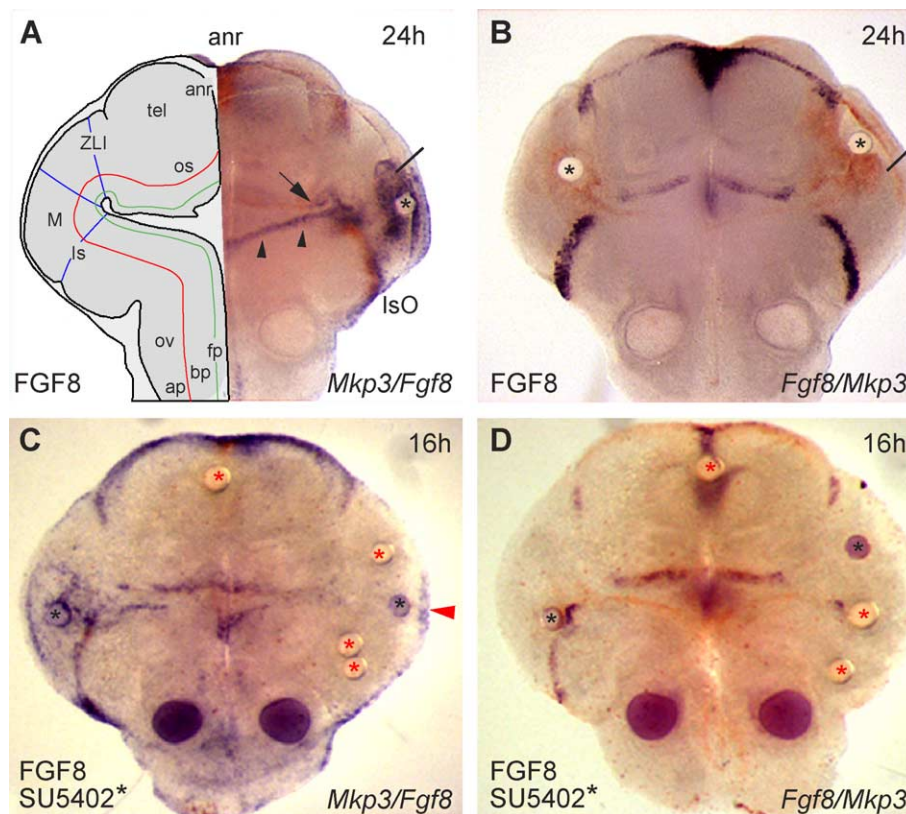

Fig. 3. *Mkp3* dependency on *Fgf8* signaling pathway. *Mkp3* is downstream of *Fgf8*. (A and B) *Mkp3* and *Fgf8* double in situ hybridization in neural tube explants of E9.5 mouse embryos 24 h after FGF8 bead implantation (indicated always by black asterisks) on both sides. (A) Ectopic induction of *Mkp3* (in dark blue color) in rostral mesencephalon. Arrowheads mark the position of the floor plate along the anterior neural tube where *Mkp3* is expressed. The black lines indicate the limit between diencephalon and mesencephalon. The arrow indicates the rostral limit of the *Mkp3* floor plate expression domain at the level of the retromammillar region. (B) Ectopic induction of *Mkp3* when the beads were placed in the diencephalon (*Fgf8* in dark-blue labeling and *Mkp3* in red). Note that no *Fgf8* ectopic induction was detected during the incubation period. (C and D) Blockage of FGF receptors with SU5402 beads (indicated always with red asterisks) after 16 h. FGF8 beads (black asterisks) are used as a positive *Mkp3* induction control. The right half of the explant is the pharmacological experimental side. (C) SU5402 rapidly inhibits the endogenous expression of *Mkp3* in the IsO and floor plate. Red arrowhead indicates the region where SU5402 has no effect upon Fgf8 signaling as is seen by *Mkp3* expression at the edge of the explant (roof plate). (D) The same situation as in C but this time the dark-blue labeling is for *Fgf8* while *Mkp3* is in red. SU5402 beads locally inhibit the expression of *Fgf8* in the isthmic organizer (IsO). Note that in the control side (left half), an FGF8 bead in the IsO has no effect on the endogenous *Fgf8* but leads to an overexpression of *Mkp3* (in red). Abbreviations: anr, anterior neural ridge; tel, telencephalon; os, optic stalk; D/M diencephalic mesencephalic boundary; fp, floor plate; ov, otic vesicle.
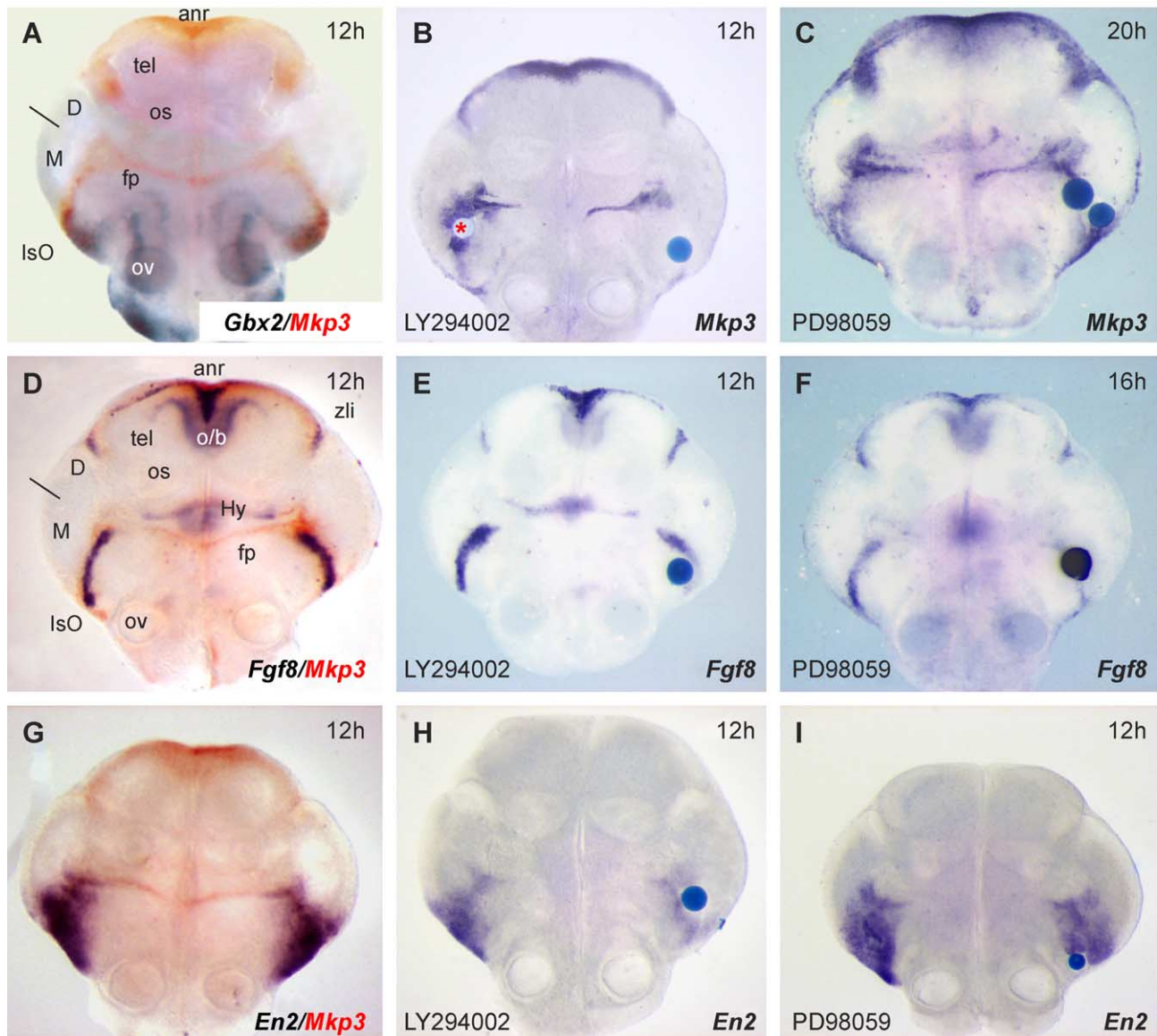
Fig. 4. Effects on *Mkp3*, *Fgf8*, and *En2* expression after blocking PI3K intracellular pathway (LY294002; B, E, and H) and Ras-MAPK intracellular pathway (PD98959; C, F, and I) in the IsO of E9.5 mouse explants. (A, D, and G) Control neural tube explants with the normal gene expression patterns of isthmic organizer genes in comparison with *Mkp3* expression pattern. After 12 h of in vitro culture, the topological and topographic coordinates of these markers are maintained. After 12 h in vitro, LY294002 beads in the IsO experimental side (right half) (B) inhibit *Mkp3* expression, (E) reduce endogenous expression of *Fgf8*, and (H) partially inhibit *En2*, a *Fgf8* downstream signal. On the other hand, PD98059 beads (C) exerted no effect on the endogenous expression of *Mkp3* even after 20 h of incubation, although it significantly reduced *Fgf8* expression after 16 h (F), without affecting the expression of *En2* after 12 h (I). The red asterisk in B is an FGF8 bead in the control side. Abbreviations: anr, anterior neural ridge; tel, telencephalon; os, optic stalk; olb, olfactory bulb; Hy, hypothalamus; D, diencephalon; M, mesencephalon; fp, floor plate; ov, otic vesicle; IsO isthmic organizer.

Mkp3 is downstream of Fgf8 signaling

Implantation of ectopic FGF8 beads at the level of the mesencephalon and caudal diencephalon were able to reproduce partially or totally the isthmic molecular patterning in mouse neuroepithelial explants and in chick in ovo experiments (Crossley et al., 1996; Garda et al., 2001; Liu et al., 1999; Martinez et al., 1999). Thus, to analyze the effect of FGF8 signaling on *Mkp3* expression in the mouse neural tissue explant model, heparin beads soaked in human recombinant FGF8 protein were

implanted in different regions of the neural tube. Our experiments showed that FGF8 beads induced ectopic *Mkp3* expression when placed in the mesencephalon (Fig. 3A; $n = 15/20$), in the diencephalon (Fig. 3B; $n = 15/22$), and in the anterior telencephalon ($n = 12/18$; data not shown; Echevarria et al., 2003). The presence of *Mkp3* mRNA was first detected 7 h after FGF8 bead implantation (Fig. 6A, control side; $n = 5/11$). Twenty-four hours after incubation, this ectopic induction increased in area and intensity around the bead (Figs. 3A, B, and 6B, control side). In some cases, an ectopic induction in the superficial
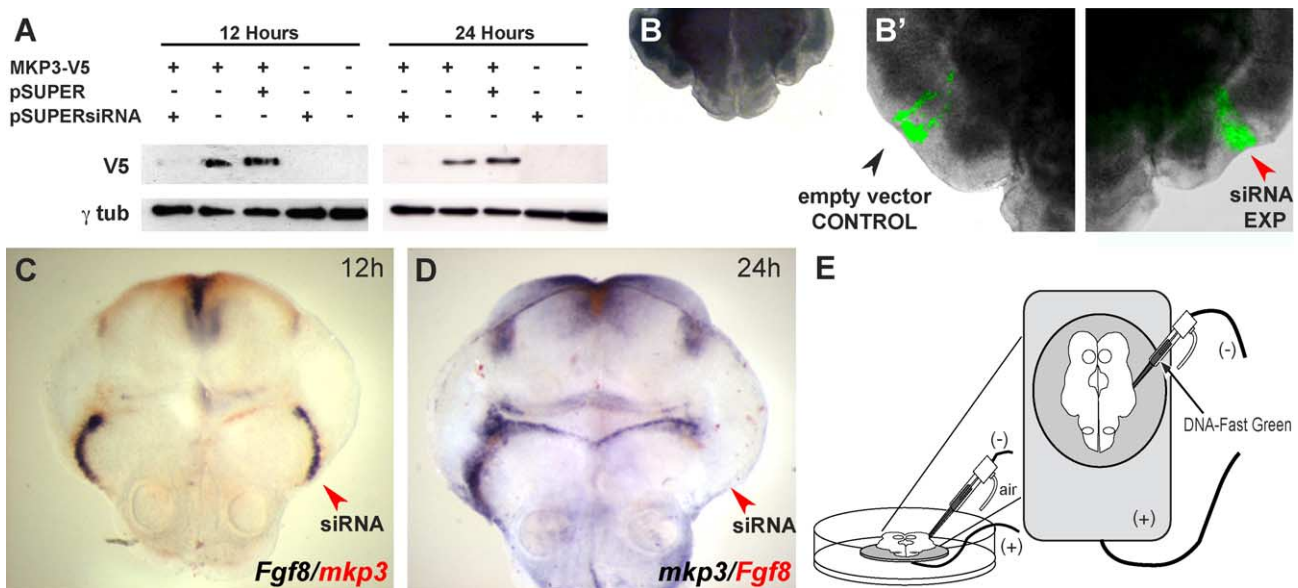
Fig. 5. Local micro-electroporation experiments with *Mkp3*–siRNA in the IsO. (A) Western blot assays proving the effectiveness of the siRNA construct in HeLa cells at 12 and 24 h after transfection. (B) An example of microelectroporation in the isthmic organizer of an explant. (B′) Magnification of B in which GFP-positive electroporated cells were specifically detected in the IsO. The right half (experimental) was used always with *Mkp3*–siRNA and the left half (control) with pSUPER empty vector. (C) Silencing *Mkp3* expression in IsO induced a clear reduction of *Mkp3* endogenous expression and also a down-regulation of *Fgf8* 12 h after microelectroporation, while the control side remained normal. (D) Total abolishment of *Mkp3* and *Fgf8* endogenous expression 24 h after microelectroporation of *Mkp3*–siRNA, while the control side remained normal. (E) Schematic representation of the microelectroporation technique used in this study (see Materials and methods). For topological coordinates, see Figs. 3A and 4A and D.

mesenchyme was also observed (see outside arrowhead in Fig. 3A). Control experiments with PBS-soaked beads never showed *Mkp3* induction (data not shown; $n = 8/8$). In none of these experiments *Fgf8* mRNA was induced by FGF8 beads in the neuroepithelium ($n = 11/11$; Figs. 3A and B; see below). In order to prove that *MKp3* induction in the neural tube is a result of Fgf8 activity, FGFR1 (a specific FGF receptor in the IsO; Trokovic et al., 2003) was pharmacologically blocked by implanting beads soaked in indolinone tyrosine kinase inhibitor SU5402 (see Montero et al., 2001; $n = 33$). SU5402 was able to significantly reduce *Fgf8* endogenous expression in the ANR and to completely abolish the transcript in the IsO ($n = 7/11$; Figs. 3C and D). Concomitantly, *Mkp3* endogenous expression was also reduced and/or abolished ($n = 6/10$) in the same places. Moreover, when FGF8 beads were co-implanted with SU5402 beads (see the experimental right half of the neural tube explants in Figs. 3C, D), the ectopic level of *Mkp3* mRNA induction around the FGF8 bead was reduced or inhibited when compared with the control side with an FGF8 bead (control left half of the explant in Figs. 3C and D; see red arrowhead in Fig. 3C; $n = 7/12$). Thus, these experiments demonstrate that in the mammalian neural tube *Mkp3* is downstream of Fgf8 signal and that Fgf8 is sufficient to activate *Mkp3* expression.

Signal transduction pathway mediating Mkp3 induction in the mouse isthmic organizer

Signaling via FGF receptors, tyrosine kinase receptors (RTK), can activate a number of downstream pathways including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K). Activation of the MAPK culminates in the translocation of extranuclear signal-regulated MAP kinases (ERK) into the nucleus and ultimately to transcriptional changes (Dickinson et al., 2002; Kouhara et al., 1997; Lin et al., 1998; Lovicu and McAvoy, 2001). Full activation of ERK requires phosphorylation of threonine and tyrosine residues by a class of MAP kinase/ ERK kinase (MEK), like MEK1 (Cowley et al., 1994; Marshall, 1995). We investigated, the potential role of the MAPK regulatory pathway in the activation of *Mkp3* expression, along the time, by blocking MEK1 with its specific inhibitor, PD98059 ($n = 43$). Beads soaked in PD98059 were implanted in the IsO of the mouse E9.5 neural tube explants (Figs. 4C, F, and I). The results showed that even after 20 h of bead implantation, the endogenous *Mkp3* transcript was still maintained around the beads (Fig. 4C; $n = 8/12$). *Fgf8* gene expression, on the other hand, was partly reduced but not abolished after 16 h (compare experimental with control side; Fig. 4F; $n = 6/12$). Other *Fgf8* downstream targets, such as *En2*, were not affected during the first 12 h (Fig. 4I; $n = 5/6$). In fact, a clear inhibition of *Fgf8*, *Mkp3*, and *En2* expression was only detected 24 h after PD98059 bead implantation ($n = 8/13$; data not shown).

It is well established that FGFs are also involved in cell survival and apoptosis through the PI3K signaling cascade (Browaeys-Poly et al., 2000; Chen et al., 2000; Ong et al., 2001). To determine whether this pathway is responsible for *Mkp3* activation by FGF8, beads soaked in the PI3K-
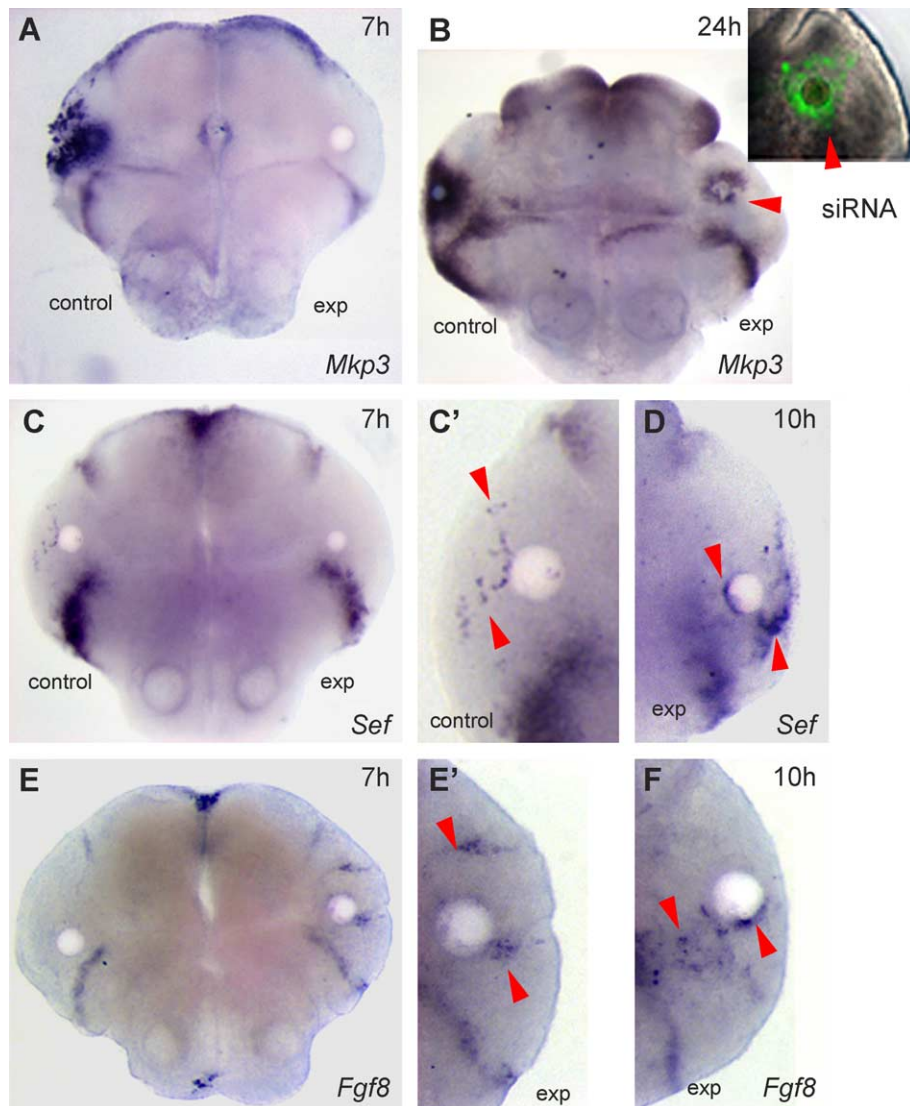
Fig. 6. Set of experimental assays using focal ectopic microelectroporation of *Mkp3*–siRNA followed by implantation of FGF8-soaked beads (experimental side; see insert in B). In the control side (left side), pSUPER empty vector was electroporated and another FGF8 bead was placed. (A) Seven hours after microelectroporation and bead implantation, an overexpression of *Mkp3* occurred around the bead in the control side, while no ectopic induction of *Mkp3* around the FGF8 bead is observed in the experimental side. (B) After 24 h, an ectopic induction of *Mkp3* in the experimental side was detected although less intense when compared to the control side. (C) *Sef* expression pattern in mouse neural tube explant and the effects when the experimental side was microelectroporated with *Mkp3*–siRNA. As seen by the magnification of panel C (C′), only in the control side a few ectopic *Sef*-positive cells start to be detected. The comparison between A and C confirms a faster and stronger induction of *Mkp3* than *Sef* in the control side by FGF8. (D) After 10 h, *Sef* expression is ectopically induced around the bead. (E and F) Ectopic induction of *Fgf8* in the mouse neural tube under these experimental conditions. As noted by the red arrowheads, the ectopic induction of *Fgf8* is clearly detected not only around the bead but also at some distance away from it. For topological coordinates see Figs. 3A and 4A and D.

inhibitor, LY294002 ($n = 35$), were implanted in the IsO. We observed that 12 h after LY294002 bead implantation, there was a total absence of *Mkp3* transcripts in the IsO (Fig. 4B; $n = 7/10$). After 12 h, only a partial inhibition of *Fgf8* was observed (Fig. 4E; $n = 6/8$), being completely abolished 20 h after bead implantation ($n = 10/11$; data not shown). Moreover, after 12 h, *En2* expression was drastically reduced in the IsO (Figs. 4G and H; $n = 5/6$). Although we cannot exclude the possibility that other pathways might be involved in the regulation of *Mkp3*, these experiments strongly suggest that *Mkp3* induction by FGF8 is mediated

mainly through the PI3K pathway since the effect through the MAPK/ERK cascade is only observed after a longer period of time. Inhibition of PI3K also reduced the expression of *Fgf8* and the downstream isthmic transcription factor, *En2* (see Fig. 7).

Functional action of Mkp3 in the mouse isthmic organizer

Given that *Mkp3* specifically binds to and blocks both the phosphorylation and enzymatic activation of ERK-2 when expressed in Cos-7 cells (Muda et al., 1996), in PC12
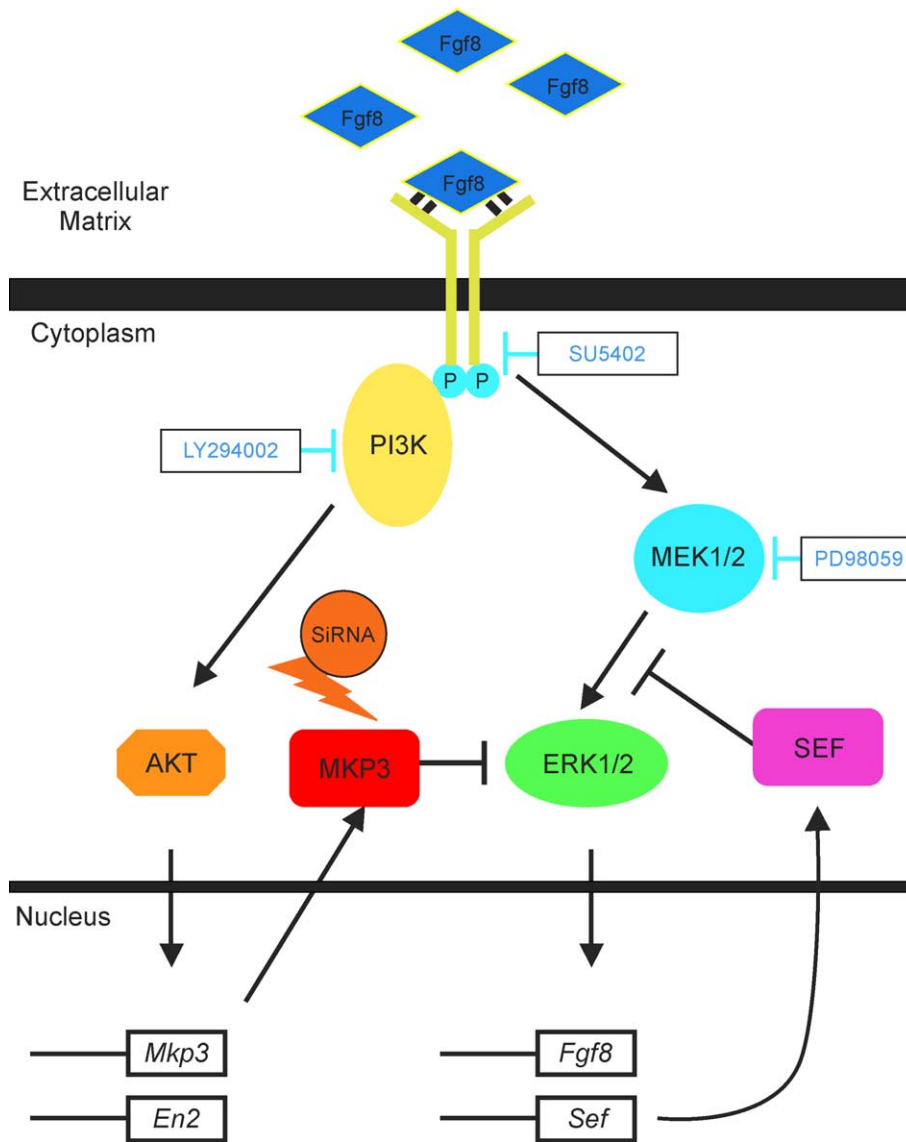
Fig. 7. Schematic representation of the experiments reported in this study and a proposed simplified model mechanism of the negative feedback loop regulation of Fgf8 intracellular signaling in the mouse isthmic organizer. Fgf8 activity must be tightly self-regulated through different intracellular signaling pathways (being Mkp3 a crucial element) in order to lead to the precise level of activity necessary for the correct patterning and development of the mammalian anterior neural tube.

cells (Camps et al., 1998), in chicken limb bud (Eblaghie et al., 2003; Kawakami et al., 2003), and also in mouse lens explants (Lovicu and McAvoy, 2001), we then asked if *Mkp3* plays the same role in the IsO. For this, we took advantage of the small interfering RNA technology (siRNA) and designed a siRNA construct against mouse *Mkp3*. First, we tested it in human HeLa cells by cotransfecting the *Mkp3* in frame with a V5-His C-terminal and the *Mkp3*–siRNA (pSUPER–*Mkp3*/siRNA).

In a first experiment, we determined the optimal DNA concentrations for the three transfected constructs that were for pSUPER-Mkp3/siRNA 1.0 μg, for pCDNA6-Mkp3/V5-HisC 0.5 μg, and for pSUPER 1.5 μg (data not shown). In Fig. 5A, we show by Western blot using an antibody against the V5 tag that 12 h after transfection, the siRNA construct

completely abolished *Mkp3* expression. We then checked if this reduction in gene expression was transient, which could restrict its application. It was observed that 12, 24 (Fig. 5A), 36, and 48 h (data not shown) after transfection, the siRNA was still almost 100% effective. This was a specific and noncytotoxic effect since on the one hand there was no visible abnormal cell death in cells transfected with the siRNA construct (data not shown), and on the other hand these cells normally expressed γ-tubulin protein (Fig. 5A). We also transfected pSUPER vector with Mkp3-V5 and saw that the empty vector had no influence on the level of expression of *Mkp3* (Fig. 5A). After these control assays, we tested the effectiveness of *Mkp3*–siRNA in the mouse neural tube explant by microelectroporating it at the level of the IsO using GFP as a targeting efficiency control (Figs. 5B

and B′). The right (experimental) side of the explant was used to drive localized expression of *Mkp3*–siRNA and the left (control) side was used to drive the empty vector alone. Based on the described time course for GFP detection and siRNA effect (see Stuhmer et al., 2002), all the following experimental assays were analyzed the earliest 7 h after electroporation. The results from these experiments ($n = 27$) show that only 12 h after electroporation, a significant down-regulation of *Mkp3* expression was detected (Fig. 5C; $n = 9/15$). Notably, when neural tube explants were analyzed 24 h after electroporation, the endogenous expression of both *Mkp3* and *Fgf8* were completely abolished in the experimental side while still unchanged in the control side (Fig. 5D; $n = 8/12$).

A hypothetical explanation for these observations might be that initially the increasing levels of ERK1/2 due to *Mkp3* inactivation by siRNA favor the expression of *Fgf8* (see Figs. 6E′, F, and 7). After 24 h, the inhibition of *Fgf8* expression (Fig. 5D) may result from an up-regulation of the pathway leading to *Sef* expression (Furthauer et al., 2002; Tsang et al., 2002) due to the levels of ERK1/2 having overcome a certain threshold. This switch of pathways would explain the disappearance of *Fgf8* expression from 7 to 24 h since the presence of *Sef* would inhibit ERK1/2 activation and therefore its pathway. Although, no experiment was done to check for *Sef* expression after 24 h, the fact that its expression starts to be detected at 10 h helps supporting the previously described hypothesis.

Mkp3 is a negative modulator of Fgf8 expression in the neural tube

In chick embryos, FGF8-soaked heparin beads induce and maintain downstream organizer genes such as *Gbx2*, *En1/2*, *Pax2-5*, and *Wnt1* (for review, see Echevarria et al., 2003) and also *Fgf8* itself. Considering that *Mkp3* is a negative feedback regulator of Fgf8 signaling in the vertebrate limb bud (see Eblaghie et al., 2003; Kawakami et al., 2003), we once again took advantage of the FGF8 bead experiments to explore if *Mkp3*-induced expression blocks Fgf8 self-activation in mouse neural tube explants. In this set of experiments, before implanting the FGF8 beads in an ectopic region of the brain explants (caudal diencephalon and/or mesencephalon), *Mkp3*–siRNA was microelectroporated (together with GFP; see insert in Fig. 6B). In this way, we generated a domain where no endogenous *Fgf8* expression is supposed to occur and where MKP3 mRNA is absent. Then the effect of FGF8 protein in the absence of *Mkp3* was analyzed at different times (7, 10, and 12 h after bead implantation, $n = 37$). As expected, 7 h after implantation no ectopic *Mkp3* induction was detected around the bead in the experimental side when compared to the control side (Fig. 6A; $n = 5/11$). After 10–12 h, a small number of cells started to ectopically express *Mkp3* at the experimental side ($n = 8/14$; data not shown). This expression became clearly detectable 24 h after bead

implantation ($n = 7/12$; Fig. 6B), although markedly reduced when compared to the strong induction in the control side. Note that the endogenous expression of *Mkp3* in the IsO (both control and experimental sides) is unaffected. We also analyzed the effect on *Sef* expression ($n = 23$) demonstrating that 7 h after FGF8-bead implantation, ectopic *Sef* expression was only partially observed in the control side ($n = 5/7$; Fig. 6C) and at very low levels, particularly surprising when compared to the high level of ectopic expression of *Mkp3* in the control side after the same time of incubation (Fig. 6A). After 10 h, the ectopic induction of *Sef* started to be clearly detected in the experimental side ($n = 4/5$; Fig. 6D). These results demonstrate that in comparison to *Sef*, *Mkp3* has a stronger response to FGF8 signal in mouse neural tube explants. The evidence of *Mkp3* as a negative feedback Fgf8 modulator was provided when FGF8 beads were able to induce *Fgf8* expression around the bead during the first 7–10 h after bead implantation in the experimental side, possibly due to an up-regulation of ERK1/2 in the absence of its inhibitor MKP3 ($n = 4/5$; Figs. 6E and F), as described above. This has never before been observed in mouse neuroepithelium (see Discussion). After 12 h, Fgf8 signal is lost ($n = 5/6$; data not shown), coinciding with the up-regulation of ectopic *Mkp3* and *Sef* expressions in the experimental side (not shown; Fig. 6D). Thus, these last experiments demonstrate that both *Mkp3* and *Sef* genes are negative feedback modulators of Fgf8 expression in the mammalian IsO, possibly through the inhibition of MAPK pathway.

Discussion

The isthmic organizer (IsO) and Fgf8 activity have been for more than a decade an optimal developmental model to study intracellular signaling mechanisms involved in processes of growth, differentiation, regionalization, and morphogenesis.

In the present paper, we demonstrate that the mouse dual mitogen-activated protein kinase phosphatase-3 (*Mkp3*) is a negative regulator of FGF signaling, in the context of early specification and determination of the mouse IsO. Recent work has provided evidence that FGF signaling is negatively regulated by complex intracellular signals in organizer centers of *Drosophila* (Hacohen et al., 1998; Wakioka et al., 2001), zebrafish, and *Xenopus* (Furthauer et al., 2002; Tsang et al., 2002), as well as in mouse embryonic fibroblast modified cell lines (Lax et al., 2002). These signals include *Sprouty*, *Sef*, *Spred*, and *FRS2-alpha*, which act through the Ras-MAP kinase pathway. More recently, in chicken limb bud development, it has been proved that *Mkp3* indeed acts as an important negative regulator of MAPK–Fgf8 intracellular signaling, induced through the PI3K intracellular pathway (Kawakami et al., 2003).

In this paper, we first confirmed that mouse *Mkp3* expression colocalizes with *Fgf8* in the neural tube, as

proposed elsewhere for *Mkp3* (Dickinson et al., 2002) and other known negative modulators (Chambers et al., 2000; Furthauer et al., 2002; Hacohen et al., 1998; Kovalenko et al., 2003; Lax et al., 2002; Liu et al., 2003; Tsang et al., 2002; Wakioka et al., 2001). We also show that the area of expression of *Mkp3* is from E9.0 onwards, wider than that of *Fgf8*, particularly at the level of the IsO and in the anterior neural ridge (ANR), two of the three known secondary organizers (Echevarria et al., 2003). *Mkp3* expression is also very strongly detected in the mesenchyme adjacent to the IsO and ANR. The wider expression domain of *Mkp3* and other known Fgf8-negative modulators, like *Sef* and *Sprouty* genes, was also described in zebrafish and mouse. This may be a consequence of the diffusion of FGF8 proteins away from the cells that originally produced it (Furthauer et al., 2002). Along the mouse neural tube, besides this *Mkp3–Fgf8* colocalization, *Mkp3* is also expressed in the floor plate of the neural tube. Expression is also found in the mesenchyme and in the somites (Klock and Herrmann, 2002), suggesting other additional roles during neurogenesis and somitogenesis.

The close relation between these two genes was first analyzed in the IsO using specific mouse knockout lines. In $Wnt1^{-/-}$, the IsO disappears at early stages (McMahon and Bradley, 1990; McMahon et al., 1992) and therefore *Fgf8* expression, which suggests the requirement of WNT1 for maintenance and stability of the midhindbrain boundary (Bally-Cuif et al., 1995). However, unlike WNT1, FGF8 is so far the only known molecule to mimic the IsO patterning activity (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). In this mutant line, we found that *Mkp3* expression was also not detected in this region. Moreover, in $Gbx2^{-/-}$ mutants, where a caudalization and a reduction of *Fgf8* expression levels occurs in the IsO (Joyner et al., 2000; Millet et al., 1999; Wassarman et al., 1997), a concomitant reduction and caudalization of *Mkp3* expression was also detected indicating a close interaction between both genes.

To further investigate the effect of Fgf8 on *Mkp3* induction, FGF8-soaked beads were implanted in ectopic locations of mouse neural tube explants. The results show that there was an induction of *Mkp3* expression around the bead, suggesting a dependency on Fgf8 signaling. This is in agreement with previous reports where FGFs were shown to induce *Mkp3*, like in PC12 cells (Camps et al., 1998), in chicken neural plate and limb bud (Eblaghie et al., 2003; Kawakami et al., 2003) and in mouse lens explants (Lovicu and McAvoy, 2001). Although in these reports the inductive effect was described to be much faster.

Mice lacking Fgf receptor-1 (*Fgfr1*), specifically in the midbrain and r1, showed that FGFR1 is the primary FGF receptor required in midbrain and cerebellum development (Trokovic et al., 2003). SU5402 is known to be a chemical blocker of FGF signal in many systems and animal models by specifically binding to FGFR1 (Eblaghie et al., 2003; Mandler and Neubuser, 2001; Moftah et al., 2002; Montero et al., 2001; Shinya et al., 2001; Walshe et al., 2002). In our experiments, locally blocking FGFR1 with SU5402 beads resulted in a complete abolishment of *Mkp3* gene expression in the IsO within 12 h of incubation and a drastic reduction in the ANR. These results together with the experiments described above suggest that FGF8 signaling is necessary and sufficient to induce *Mkp3* in the mouse neural tube. Although we cannot exclude the possibility that other FGFs, like Fgf17 and Fgf18 (Liu et al., 2003), may be involved in *Mkp3* induction since they are also induced by Fgf8 (Chi et al., 2003), have a similar temporal and spatial expression domain, and like Fgf8 signal through FGFR1.

Among the different signaling transduction pathways that may be activated by FGFs are the MAPK and the PI3K pathways (Javerzat et al., 2002). To investigate which is the pathway responsible for *Mkp3* induction, specific inhibitors for the classical MAPK (MEK1/2 inhibitor; PD98059) and PI3K kinase (LY294002) pathways were used. The results show that maintenance of *Mkp3* endogenous expression in the IsO by FGF8 occurs mainly through the PI3K signaling cascade rather than the MAPK pathway. Indeed, abolishment of endogenous *MKp3* took place within the first 12 h after LY294002 bead implantation. In addition, a severe reduction of *Fgf*–IsO downstream genes such as *En2* also occurred, suggesting that the expressions of both *Mkp3* and *En-2* are transcriptionally induced through the PI3K pathway and indicating the importance of PI3K as part of the Fgf8 pathway in the regulation and maintenance of isthmic activity. In contrast, PD98059 after the same time of bead incubation exerted no effect on the endogenous expression of *Mkp3*. A slight decrease on *Mkp3* expression was only observed a few hours later. A similar result was observed for *Fgf8* expression after LY294002 treatment. These results suggest a crosstalk between the PI3K and MAPK signaling pathways, as has been seen in a number of systems for receptor tyrosine kinase signaling networks. It is a generally accepted hypothesis that Fgf8 through MAPKs (ERK1/2) normally stimulates cell proliferation, differentiation, and apoptosis in the IsO (Dickinson et al., 1994; Wurst and Bally-Cuif, 2001; Ye et al., 2001). While Fgf8, through PIK3, performs an essential role in cell survival (Chen et al., 2000; Ong et al., 2001; Powers et al., 2000), as shown in the Fgf8 conditional knockout in which the IsO deletion is due to massive cell death during early neurogenesis (Chi et al., 2003). A balance between these two pathways has to be present to maintain the morphological and functional characteristics of the IsO. Among others, *Mkp3* seems to be involved since it is activated by one pathway (PI3K) and negatively regulates the other (ERK1/2). Actually, sustained domains of ERK activation levels have been observed in the mouse neural tube, predominantly in the IsO and ANR, limb bud, and tail bud (Corson et al., 2003); and, as described, *Mkp3* is a specific and potent inhibitory regulator of the ERK class of MAP kinases (Camps et al., 1998; Groom et al., 1996; Muda et al., 1996). Local inactivation of *Mkp3* in the chicken limb bud results in a significant

increase of ERK activation (Eblaghie et al., 2003; Kawakami et al., 2003). Thus, it may be assumed that *Mkp3* has the same function in the mouse IsO (Fig. 7). Our results show that *Mkp3* induction is dependent of *Fgf8* signaling during normal isthmic organizer activity. This induction/maintenance is guided mainly through the PI3K cascade pathway (a survival/apoptosis pathway; Chen et al., 2000; Ong et al., 2001; Powers et al., 2000), involved in the control of ERK activation (Liu and Joyner, 2001a,b; Nakamura, 2001) through Mkp3, which together with the PI3K pathway are important for mesencephalon/rhombencephalon development.

Loss-of-function experiments using siRNA aimed to silence endogenous *Mkp3* expression through focal gene transfer using a microelectroporation technique in the mouse isthmic region showed the complete abolishment of endogenous *Mkp3* expression and also *Fgf8* expression within the 24 h that lasted the in vitro experiment. These unexpected results suggest a collateral activation of *Sef*-negative regulatory pathway. In fact it was seen that 10 h after the siRNA effect, *Sef* starts to be expressed, which coincides with FGF8 downregulation after 12 h. The induction of *Sef* expression may be involved in the abolishment of Fgf8 expression after 24 h, since as has been reported it acts as a feedback-induced antagonist of Ras/MAPK-mediated FGF signaling (Furthauer et al., 2002). However, we cannot exclude the possibility that other genes belonging to the same synexpression group and expressed in the same tissue, like the *Sprouty* genes, may also have a role in this effect (Chambers, 2000; de Maximy et al., 1999; Furthauer et al., 2002).

The regulation of *Fgf8* expression by *Mkp3* was demonstrated when we ectopically electroporated *Mkp3*–siRNA in a location of the neural tube where neither *Mkp3* nor *Fgf8* are normally expressed present. After implanting an FGF8-soaked bead, we saw that FGF8 is capable of inducing its own mRNA after a short period of time (within the first 7 h) in the absence of *Mkp3*, an unraveled issue in the mammalian neural tube development (Garda et al., 2001; Liu et al., 1999).

The finding that in the mammalian neural tube FGF is indeed only capable to induce *Fgf8* expression after blocking *Mkp3* might suggest an evolutionary differential control mechanism by *Mkp3* on Fgf8 signaling. In fact, in contrast to the mouse, the onset of *Mkp3* expression in the chick embryo is delayed from that of *Fgf8* expression (C. Vieira and S. Martinez, unpublished results). This indicates that different developmental time window activation mechanisms of both signals during early development can play an important role in neural tube patterning among different species of vertebrates.

FGF signaling pathways in the chicken limb bud and in the mouse IsO seem to differ in the contribution of the PI3K vs. MAPK pathways. In the chicken limb bud, it was proved that the PI3K and the MAPK pathways were mutually exclusive in the mesenchyme and the apical ectodermal ridge (AER),

although being both activated by FGF8 produced in the AER. This exclusiveness was accomplished by antagonists, namely, MKP3 that completely abolished the MAPK pathway effector pERK in the mesenchyme where it is expressed (Kawakami et al., 2003). In fact in the chick limb bud, the induction of *Mkp3* was so strong and quick that possibly prevented pERK from being activated in the same tissue. In the mouse IsO, a different situation seems to occur; in fact in this tissue, *Mkp3* is expressed and pERK is also present (data not shown). So in this tissue, both PI3K and the MAPK pathways seem to be active. It is also true that the induction of *Mkp3* is not as quick as in the chick, and this may be the reason why both pathways can coexist in a steady-state level and modulate tightly each other signaling. The coexistence of both pathways has been described previously in the mouse neural plate where a high level of expression of *Mkp3* correlates with active MAPK pathway (Eblaghie et al., 2003), in *Xenopus* where both pathways work in parallel in mesoderm induction (Carballada et al., 2001), and possibly in zebrafish (Tsang et al., 2004).

In many model systems, it has been reported that *Mkp3* expression is maintained by active FGF signaling through the Ras/MAPK pathway. This is the case in *Xenopus* where *Mkp3* expression has been shown to be directly induced by retinoic acid (RE; Mason et al., 1996; Moreno and Kintner, 2004). Also in zebrafish, the early expression of *Mkp3* is proposed to be directly induced by maternal β-catenin (Tsang et al., 2004). In chick, both PI3K and MAPK pathways have been described to induce *Mkp3* expression, in the limb bud and neural plate, respectively (Eblaghie et al., 2003; Kawakami et al., 2003). In the mouse IsO, as in the chick limb bud, *Mkp3* seems to be induced by PI3K. All these reports increasingly suggest that the regulation of *Mkp3* by FGF signaling in different environments may be under different signaling cascades.

In conclusion, we have demonstrated that in the isthmic region of the mouse embryo, Fgf signaling must be tightly self-regulated through different intracellular signaling pathways (being Mkp3 a crucial element) in order to maintain the correct balance in the intracellular signaling network necessary for the patterning and development of the mammalian neural tube, particularly the mesencephalon and rhombencephalon.

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